

ASCI OF THE OPERCULATE DISCOMYCETES (PEZIZALES)

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF  
THE UNIVERSITY OF FLORIDA  
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE  
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1977

## ACKNOWLEDGEMENTS

I wish to express gratitude to Dr. James W. Kimbrough, Chairman of the Supervisory Committee, for his guidance, friendship and patience throughout the course of my study. I am also indebted to Dr. Henry C. Aldrich and the staff of the Ultrastructure Laboratory for the use of the equipment and facilities and for their appreciative assistance.

I wish to sincerely thank D. G. Griffin, III, for his considerate advice and encouragement over the last five years. I also wish to extend my thanks to Drs. D. A. Roberts and N. C. Schenck, as members of my Supervisory Committee along with Drs. Kimbrough, Aldrich and Griffin, for their helpful comments and suggestions.

Sincere appreciations are accorded to Dr. G. L. Benny and other researchers associated with the Mycological Laboratory and for their encouragement and assistance.

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Abstract of Dissertation Presented to the Graduate Council  
of the University of Florida in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

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by

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December, 1977

Chairman: James W. Kimbrough

Major Department: Botany

In the taxonomy of Ascomycetes greater emphasis is being placed on ascal structure and the mechanism of spore release. All components associated with ascal dehiscence are collectively referred to as the apical apparatus. Within the operculate Discomycetes (Pezizales) ascal characters, including the apical apparatus, have provided significant contributions in the delimitations of families and genera.

The primary purpose of this research was to understand more clearly the nature of the operculate apical apparatus and to determine whether this character can be implemented as a systematic tool. To do this, ascal tips of approximately 30 species, representing 26 genera, were examined with the aid of the light and electron microscopes. The stains, Congo red, acid fuchsin, aniline blue, Melzer's reagent and toluidine blue were commonly used for light microscopy, while lead citrate, uranyl acetate and silver methenamine were used for electron microscopy. Attention

was paid to morphology, development and cytochemistry of the apical apparatus as a whole, to peculiar structures, and to the time of their appearance during ascosporogenesis.

Morphological, cytochemical and ultrastructural evidence revealed that the apical apparatus fell into six groups. (1) Members typically with iodine-positive asci, including species of Peziza, Ascobolus, Saccobolus and Thecotheus, possess annular indented zones of dehiscence. Exogenous mucilaginous coats are believed to be responsible for the blue reaction. Although Iodophanus is iodine-positive, very few properties of this taxon are shared with those of the annular indented species or any other member of the Pezizales. (2) Eugymnohymenial discomycetes such as Pyronema, Ascodesmis and Coprotus have ascal walls which taper in thickness at the tip. Wide zones of dehiscence are formed in the outer layer of Coprotus and Ascodesmis. In Pyronema the outer layer of the operculum is differentially stained from the rest of the ascal wall. (3) Species placed in either the Otideaceae or the Aleuriaceae lack conspicuous dehiscent zones. The presence of subapical rings in Aleuria, Anthracobia, Ascozonus, Humaria, Jafnea, Otidea, Scutellinia and Sphaerosporella distinguish the ascal tips of these taxa from the rest of the operculate Discomycetes. (4) Members with large, variously shaped apothecia, i.e., Morchella and Helvella, possess distinct zones of dehiscence in the inner layer. Mature lateral walls vary little in thickness from the apical walls.

Opercular delimitation with the light microscope is observed in Rhizina and Discina when stained with Congo red.

(5) Four species of Thelebolus exhibit thick-walled apical domes that are subtended by distinctly stained rings. Wall structure most closely resembles that of the bitunicate ascus. The inner layer consists of microfibrils organized in a banded pattern. Ascal dehiscence occurs in a modified jack-in-the-box manner. (6) Trichobolus zukaii possesses a large operculum which is functionally inoperative. The ascal wall is heavily stratified and lacks distinctive features.

The apical apparatus of the operculate ascus has marked variability. At least four major forms exist. In addition two variations are found in Trichobolus and Iodophanus and may be considered as separate forms. The apical apparatuses of Thelebolous demonstrate very few microchemical, developmental and morphological similarities with those found in the other species. The taxonomic placement of Thelebolus within the Pezizales is in serious doubt. Examination of the apical apparatus can be useful as a systematic tool in helping resolve ambiguous taxonomic relationships and determine phylogeny within the Pezizales.

## GENERAL INTRODUCTION

For the past century ascal characters have played an increasingly significant role in Ascomycete taxonomy. The early work of the Crouan brothers (1857), Nylander (1869) and Fuckel (1869) revealed notable differences in the ascus with respect to size, shape, microchemical properties and mode of spore release. Shortly thereafter, a broad scheme of Discomycete classification was proposed (Boudier, 1879, 1885), the first in which ascal features were exploited. The significance of this concept has not been fully appreciated until recently.

During the last 35 years, ascal structure has been recognized as one of the fundamental diagnostic characters within the Ascomycotina. Studies by Chadeaud (1942) and Miller (1949) on asci within the Discomycetes and Pyrenomycetes revived interest in this area of taxonomic research. Miller (1949) used various features, including wall thickness, dimensions of the ascus, and presence or absence of a pore or cap at the ascal apex to separate major orders and families of the Pyrenomycetes. Chadeaud (1942, 1946) focused mainly on the tip of the ascus in both the Pyrenomycetes and Discomycetes. He paid particular attention to all components directly involved in ascospore



expulsion. He used the term "apical apparatus" in defining that region of the ascus. In addition to these works, Luttrell (1951) separated the Pyrenomycetes into two groups, the unitunicates and the bitunicates, basing the division on essential structural features of the ascal wall.

Within the discomycete order Pezizales, ascal characters have provided an important contribution for the distinction of families, subfamilies and genera (Eckblad, 1968; Kimbrough, 1970; and van Brummelen, 1974). Typically, the ascospores are liberated through a circumscissile rupture, the operculum, at the tip of the ascus. Most studies concerned with dehiscence of the ascus have been restricted to representatives within the smaller suborder Sarcoscyphineae (Chadefaud, 1946; LeGal, 1946a, 1946b, 1953; van Brummelen, 1975; Samuelson, 1975). LeGal and Chadefaud noted distinct characters that appeared similar to the apical apparatus of various members within the inoperculate Discomycetes. They proposed a phylogenetic relationship between representatives of the Sarcoscyphineae and certain representatives of the Helotiales. Recent ultrastructural studies (van Brummelen, 1975; Samuelson, 1975) demonstrated that all asci were truly operculate in six genera of the Sarcoscyphineae. Marked differences were described in the dimensions of the bilayered ascal wall surrounding the operculum. Samuelson (1975) structurally defined the structure of this region as the suboperculum and noted two basic forms, eccentric and noneccentric. The two forms, however, were shown to be

quite variable, representing intergrades that linked the two extreme examples, Cookiena sulcipes (Berk.) Kuntze and Pseudoplectania nigrella (Pers. ex Fr.) Fuckel. Taxa that had the noneccentric form displayed significant features that were in common with the apical apparatus of an operculate species, Ascobolus stercorarius (Bull. ex St. Amans) Schroet (Wells, 1972). Phylogenetically, ascal dehiscence within the Sarcoscyphineae is believed to be affiliated most closely with that observed in the true operculates (Pezizineae).

Relatively few investigations related to mechanisms of ascal dehiscence have been made on representatives of the Pezizineae. The first comprehensive morphological study on true operculate apical apparatuses was carried out by Chadeffaud (1942). While describing the apical apparatuses in detail, he pointed out many similarities between operculate and nonoperculate asci. Chadeffaud (1942) designated a fundamental apical apparatus that contains components ubiquitous among discomycete and pyrenomycete asci. Many of these components were incorporated within a majority of the operculate apical apparatuses. In basipetal sequence they included (1) an operculum, (2) a subapical 'bourrelet' or cushion, and (3) an apical punctation or depression that led to a funnel which was attached contiguously to a tract that led to the base of the ascus. However, other elements that were depicted in the fundamental apical apparatus were not reported for operculate asci. Chadeffaud (1942)

theorized that the asci of these fungi have become modified through the process of evolutionary regression.

In a recent systematic treatment of the ascus, Chadeffaud (1973) separated ascus types into three categories, the *Archaeascès*, the *Nassascès* and the *Annelascès*. The division was based both on ascal wall structure and the presence and absence of principal components that comprised the apical apparatus. He retained the view that a fundamental unit of ascus structure exists. The prototype apparatus depicted in his earlier study (1942) is basically similar to the type depicted for the *Annelascè*. Chadeffaud (1960, 1973) renamed the region of the "coussinet apical" and "pontuation apicale" of the operculate asci to be "pendentif" and "chamber oculaire," respectively.

The remainder of the studies related to the structure of operculate apical apparatuses have been made primarily within the last decade. Of these, van Brummelen's (1974) work with *Ascozonus woolhopensis* (Berk. and Br. apud Renny) Hans. was the only one specifically concerned with the dehiscence apparatus. The number of investigations made on the apical apparatus of inoperculate Discomycetes (Bellèmere, 1969, 1975; Campbell, 1973; Corlett and Elliott, 1974; Schoknecht, 1975) and Pyrenomycetes (Beckett and Crawford, 1973; Greenhalgh and Evans, 1967; Griffiths, 1973; Reeves, 1971) have been considerably greater. The apparent lack of interest in the operculate apical apparatus may reflect an attitude of many investigators that most operculate apical

structures appear identical. Several previous findings suggest that the opposite may be true. In a study on ascus and ascospore development of Ascobolus streccorarius, Wells (1972) discovered that as the spores approached maturity an indented circular band was formed at the tip of the ascus, delimiting the operculum. Schrantz (1970) demonstrated in his work with Peziza and Tarzettia that the ascal apex consisted of a thicker outer layer, a feature not reported previously within the Pezizales. The study of Ascozonus woolhopensis by van Brummelen (1974) showed that the inner layer of the ascal tip initially became swollen before the layer underwent a process of localized disintegration which was followed by spore release. The data of these researchers reveal remarkable differences between dehiscence mechanisms of the selected taxa.

Microchemical properties of apical apparatuses within the Pezizales have displayed a variety of differences between numerous species. Characteristically, within the Sarcoscyphineae the inner layer of the ascus is deeply stained in Congo red, and in certain members the opercula have been shown to be microchemically distinct from the surrounding wall of the ascus (LeGal, 1946a; Samuelson, 1975). Cytochemical observations of representatives within the true operculates have been more useful for taxonomy. The most widely applied stain has been Melzer's reagent, an iodide solution that produces typically a blue reaction (Korf, 1973). In the genus Peziza (Dill.) L., taxa were

distinguished by the variable staining of the ascal tips. Nylander (1869) was one of the first to use the iodine reaction as a method for generic and specific delimitation. In other operculate representatives, Iodophanus Korf, Thecotheus Boud., and Psilopezia Berk., entire asci have been reported to be diffusely stained (Eckblad, 1968; Kimbrough, 1969 and 1970). Within the largely coprophilic family Thelebolaceae (sensu Kimbrough, 1970), asci in a number of taxa have been observed to rupture irregularly upon spore release. Uniascal, multispored forms of Thelebolus and Trichobolus appeared to have more pronounced structural features associated with their apical apparatuses. Similarly, Kimbrough (1974) discovered in the genus Lasiobolus that the uniascal species L. monascus Kimb. forms a chemically differentiated band below the apex of the ascus and that similar though less notable structures exist in eight-spored species. In Caccobius, he noted that the tip of the ascus contains a distinct, apical plug which stains in Waterman's blue black ink but remains hyaline in Congo red (Kimbrough, 1972). Conversely, preparations using Congo red and acid fuchsin in lactic acid have demonstrated the presence of a prominent apical ring located within the multi-layered ascal wall of Thelebolus (Kimbrough, 1972).

Until recently, little attention has been paid to the ultrastructure or cytochemistry of the structures associated with dehiscence of the ascus. Consequently, these features have played a small role in the taxonomy of operculate



Discomycetes. Increasing information on ascal walls and ascal tips, in particular, suggests the strong possibility that apical apparatuses within the Pezizales are significantly diverse and can be implemented as a useful systematic tool. This study presents a comprehensive survey of the apical apparatus within the Pezizineae, the primary concern being with morphological and cytochemical features. The investigation includes a conspectus of apical apparatuses on family, subfamily and tribe levels, using one or more major representatives from each taxon. Over 30 species, representing 26 genera, were inspected throughout the course of the study. Instead of attempting to describe all of the species at one time, it has been more feasible to segregate them into morphological groups. Special attention was paid to the development of the apical apparatus as a whole and to peculiar structures and the time of their appearance during ascosporogenesis. Chapter I examines the asci and the apical apparatus of representatives that are associated with the iodine-positive reaction. Included are species of Peziza L. per St. Amans, Iodophanus, Ascobolus Pers. per Hooker, Saccobolus Boud., and Thecotheus Boud. Chapter II studies the apical apparatuses of members found throughout the largest of all operculate groups, the Pyronemataceae (sensu Korf, 1973), a group which has been referred to here as the Otidea-Aleuria complex. Species of Otidea (Pers.) Bon., Jafnea Korf, Humaria Fuckel, Sphaerosporella (Svr.) Svr. & Kub., Aleuria Fuckel, Anthracobia Boud., Scutellinia

(Cooke) Lamb., Ascozonus (Renny) Hansen, Sowerbyella Nannft., and Geopyxis (Pers.) Saccardo were used. The apical apparatuses of representatives of Ascodesmis van Tieghem, Coprotus Korf & Kimb., and Pyronema Carus are described in Chapter III. Characters not found in the apical apparatuses of other representatives shared by the three members have resulted in the formation of this grouping. In Chapter IV, the apical apparatus of the Morchella-Helvella group are examined. This group represents members which typically form the largest ascocarps within the operculate Discomycetes. Species of Morchella St. Amans, Helvella L., Gyromitra Fr., Discina (Fr.) Fr., and Rhizina Fr. per Fr. were used. Chapter V examines the ascus walls and the associated apical apparatuses of four species of Thelebolus Tode per Fr. Dehiscence mechanism, wall layering, ontogeny and cytochemistry separate this genus from all members of the operculate Discomycetes. Similarly, Trichobolus zukaii Heimerl is treated by itself in Chapter VI.

The objective of this study was to learn the nature of the operculate apical apparatus and to observe what phylogenetic correlations can be made within the Pezizales.

CHAPTER I  
MORPHOLOGY, DEVELOPMENT AND CYTOCHEMISTRY OF THE APICAL  
APPARATUSES OF REPRESENTATIVES WITH IODINE-POSITIVE ASCI

Introduction

A number of workers have paid close attention to ascal features as an additional means to help classify individual representatives and major groups within the Ascomycetes. Of these features, the ascal wall and its associated mechanism of spore release have been of particular interest. (See General Introduction.)

Within the operculate Discomycetes, early workers (Nylander, 1869, Karsten, 1869; Rhem, 1896; and Boudier, 1905-1910) demonstrated the occurrence of iodine positive or "amyloid" asci in a number of species, and by 1907 (Boudier), this feature had been applied systematically along tribal lines. Recently, Kohn and Korf (1975) have pointed out that the term "amyloid," used traditionally for the positive blueing reaction of iodine-treated material, should be avoided. It implies the presence of amylose or amylose-related substances when little has been known of its chemical specificity. The present study has taken account of their suggestion and has used the terms "iodine positive" and "blueing in iodine" in place of amyloid.



During the last decade, Rifai (1968), Kimbrough (1970) and Korf (1973) have placed all members of the Pezizineae that have the "amyloid" property into two families, the Ascobolaceae and Pezizaceae. Of the genera included in these families, only Ascobolus has been shown to have species that do not turn blue in iodine. Even in this genus approximately 50% of the species were observed to give an iodine positive reaction (van Brummelen, 1967). Except for Peziza, the blueing reaction has been found to occur diffusely over the entire wall of the ascus. In Peziza, many species are distinctly iodine-positive at the apex. In certain species such as P. vesiculosa Bull. ex Fr., the blueing is restricted to a ring while the others as much as one-half of the ascus becomes stained.

Until Chadeffaud's (1942) extensive examination of the apical apparatuses found throughout the Euascomycetes, no one had investigated carefully the mechanism of dehiscence of the representatives in the Ascobolaceae and Pezizaceae. Working with material that had been treated with a chromo-osmic solution, Chadeffaud described the asci of Peziza echinospora Karst. (= Aleuria umbrina Boud.) to have a full complement of dehiscent components as described in the General Introduction. Furthermore, he stated that in all of the species which have a completely developed apical apparatus, including P. echinospora, the formation of the apical apparatus occurred before ascosporeogenesis. His observations of living material of Ascobolus furfuraceus Pers. ex

Fr. revealed the presence of a modified apparatus which lacked a distinct funnel and tract. He depicted the ascal tip as having a remarkably differentiated opercular dome and subtending pad (Fig. 51a). Consequently, he reasoned that *Ascobolus* was a very specialized group which represented a line of evolutionary regression.

Although Moore (1963), Reeves (1967) and Carroll (1969) had previously studied the fine structure of operculate asci, Schrantz (1970) was the first to examine the ultra-structure and cytochemistry of the ascal walls of several ascomycetous representatives, including *Peziza plebeia* (LeGal) Nannf. (as *Galactinia plebeia*). He described the ascal wall of *P. plebeia* as consisting mostly of a thick, "chitinous-callosic" inner layer, which became thinner towards the apex, and a thin, "pectic-amyloid" outer layer, which conversely thickened at the apex (Fig. 51b). His electron microscopic examination demonstrated the two layers, thus reinforcing the light microscopic observations. He was unable, however, to distinguish from each other the "amyloid" and the "pectic" coats, which formed the outer layer.

Wells (1972) in his work with the ascus and ascospore ontogeny in *Ascobolus stercorarius* (Bull. ex St. Amans) Schroet. showed that towards the end of spore maturation an annular indentation was formed at the tip, delimiting the operculum. Simultaneously, the inner layer of the ascal wall increased in thickness throughout the apical region. Wells' and Schrantz' accounts of the ascal tips of

A. stercorarius and Peziza plebeia, respectively, displayed little in common. In fact, their findings supported Chadeffaud's belief that the two genera are not closely related.

Similarly, Eckblad (1968) concluded that Ascobolus and related genera have little phylogenetic connection with the Pezizaceae but instead are more affiliated with members of the Thelebolaceae. He suggested that the "amyloid" reaction was not a valid phylogenetic trait. By putting great taxonomic value on clavate form and protruding nature of mature asci, he felt justified in placing Ascodesmis in the Ascobolaceae and removing Iodophanus to the Pyronemaceae and Thecotheus to the Thelebolaceae (Table 1).

The usefulness of the iodine test for systematic schemes is seriously in doubt. This study examines morphological, developmental and cytochemical aspects of the iodine-positive ascus and its associated mechanism of dehiscence in order to learn whether other characteristics of the ascal wall will support or argue against the systematic use of the iodine test. Five members of the Pezizaceae and Ascobolaceae (sensu Rifai, 1968; Kimbrough, 1970; Korf, 1973) are used. They are Iodophanus granulipolaris Kimbrough, Thecotheus pelletieri (Crouan) Boud., Ascobolus crenulatus Karst., Saccobolus depauperatus (Berk. and Br.) E. C. Hansen and Peziza succosa Berk. Phylogenetic and ontogenetic comparisons are made between the species.

Chapter I  
Table 1

Classifications of Genera with Iodine-positive Asci

Eckblad (1968)

Ascobolaceae

Ascoboloideae

Ascobolus  
Saccobolus

Ascodesmidoideae

Ascodesmis  
Boudiera  
Svrekia

Pezizaceae

Peziza  
Plicaria  
Marcelleina  
Psilopezia  
Discomycetella

Thelebolaceae

Thecotheus

Pyronemaceae

Iodophanus

Korf (1973)

Ascobolaceae

Ascoboleae

Saccobolus  
Ascobolus

Iodophaneae

Boudiera  
Sphaerosoma  
Iodophanus  
Thecotheus

Pezizaceae

Gelatinodiscus  
Sacrospheera  
Pachyella  
Peziza (= Plicaria)

Rifai (1968)

Ascobolaceae

Ascobolus  
Saccobolus

Pezizaceae

Peziza  
Plicaria  
Boudiera  
Sarcosphaera  
Pachyella  
Gelatinodiscus  
Thecotheus  
Iodophanus  
Sphaerosoma

Kimbrough (1970)

Ascobolaceae

Ascoboloideae

Ascobolus  
Saccobolus

Ascodesmidoideae

Ascodesmis  
Boudiera  
Svrekia

Pezizaceae

Peziza  
Plicaria  
Sarcosphaera

Table 1 (Cont'd.)

## Pezizaceae

Pachyella  
Iodophanus  
Thecotheus  
Psilopezia

## Materials and Methods

### Collection and Developmental Determination of Material

Young and mature apothecia of Peziza succosa were collected from a sandy basin near the Devil's Millhopper in Gainesville, Florida. Specimens of Thecotheus pelletieri and Saccobolus depauperatus were found on cow dung collected near Gainesville. A culture of Ascobolus crenulatus, no. D-1476, was obtained from the Culture Collection of the Rancho Santa Ana Botanic Garden, courtesy of R. K. Benjamin. Iodophanus granulipolaris was isolated from cow dung collected near Gainesville by J. Milam. Apothecia of A. crenulatus and I. granulipolaris were grown on DOA (Dung-Oatmeal Agar; Benedict and Tyler, 1962) and WSHDD (Weitzman Silva Hutner medium with dung decoction) respectively. Portions of the apothecia of P. succosa and A. crenulatus were free-hand sectioned for light microscopic observation to determine the stage of ascal development. The mature and immature stages were then separated for further examination. Apothecia of different developmental stages of T. pelletieri and S. depauperatus were removed from the substrate and placed in thin layers of solidifying water agar and temporarily refrigerated. Whole squash mounts of individual apothecia of I. granulipolaris were used to determine the stage of ascal ontogeny for the majority of the apothecia in a particular Petri plate.

### Procedures for Light Microscopic Observations

Three to five millimeter squares from the apothecia of P. succosa and entire apothecia of A. crenulatus were placed in a 40% mucilage mixture, frozen and sectioned with a cryostat at a thickness of approximately ten micrometers ( $\mu\text{m}$ ). Sections were mounted on a drop of Melzer's reagent (Korf, 1973) on a slide and viewed with a Zeiss microscope. The Melzer's reagent was used as a general differential stain. More importantly, this reagent tested for the iodine-positive or "amyloid" reaction at the tip or throughout the ascus wall. The Congo red stain (Samuelson, 1975) was also used frequently for comparison. Similarly, whole ascocarps of T. pelletieri, S. depauperatus and I. granulipolaris were squash-mounted in either Melzer's reagent or Congo red. Less frequently, 1.0% lactophenol cotton blue, 2.0% aqueous phloxine and 2.0% analine blue in 50% glycerine solution were used to observe cytoplasmic detail and any notable change in wall morphology.

Plastic embedded material was sectioned at 0.5 to 1.0 micrometers with glass knives. Two or three sections were placed on a small drop of 0.01% sodium borate and dried at 60°C. Single drops of either 0.25% aqueous toluidine blue (Stevens, 1966) or 1.0% aqueous crystal violet were applied for 30 seconds and carefully rinsed with water before being mounted in immersion oil.

### Procedures for Electron Microscopic Observations

Five-millimeter squares of apothecia of P. succosa, entire apothecia of A. crenulatus and I. granulipolaris, and



agar blocks, containing six to eight apothecia each, of S. depauperatus were fixed in buffered (0.2 M sodium cacodylate pH 7.2) 2.0% glutaraldehyde and 2.0% paraformaldehyde solution (Karnovsky, 1965) for two hours at room temperature (= 23°C). Agar blocks containing apothecia of T. pelletieri were fixed in 1.0% permanganate solution for one hour at room temperature. All materials were rinsed 3 times during a 30-minute period in 50% buffer-50% distilled water solution and postfixed in 1.0% osmium tetroxide for one hour at room temperature. The materials were washed several times with 0.1 M sodium cacodylate buffer and dehydrated in an ethanol series (25% steps). Specimens were stained with 2.0% uranyl acetate in 75% ethanol overnight at 4°C. They were washed twice with acetone for one hour and subsequently infiltrated and embedded in a low-viscosity resin (Spurr, 1969). Three changes of 100% plastic were made to ensure removal of any residual acetone. The materials were placed under vacuum for five minutes to remove bubbles and then polymerized for one day at 60°C.

Ultrathin sections were cut on a Sorvall MT-2 ultramicrotome with a diamond knife and placed on single-hole, formvar-coated grids. Sections were normally poststained in 0.5% uranyl acetate for 15 minutes and in lead citrate (Reynolds, 1963) for 5 minutes. In addition, wall demarcation was further enhanced by posttreating unstained sections with silver methenamine, a preferential stain for polysaccharides (Martino and Zamboni, 1967). Ultrathin sections



were then examined with an Hitachi HU-11 E electron microscope.

### Results

Within the operculate ascus, three structural components, the operculum, the region or zone of dehiscence, and the adjacent lateral walls referred to as the suboperculum (Samuelson, 1975), collectively comprise the apical apparatus. The apical apparatus of each species differs to some degree developmentally and in size and shape. For clarity, the mechanism of dehiscence for each representative will be treated individually.

#### The Apical Apparatus of *Peziza succosa*

The diploid ascus reaches a length of 140-180  $\mu\text{m}$  and a diameter of 10-12  $\mu\text{m}$ . In Melzer's reagent a deep blue reaction occurs and is restricted to the immediate region of the apex (Fig. 1). The outline of the tip of the ascus appears to be rather uneven, and the cytoplasm of that region has a distinctly granular appearance. Fully mature asci reach a length of 200-210  $\mu\text{m}$  and a diameter of 12-14  $\mu\text{m}$ . When stained with Melzer's reagent, the deep blueing at the tip is less intense and extends 10-14  $\mu\text{m}$  down the sides of the ascus wall (Fig. 2). The outline of this region is smooth at this time. On occasion, a blue staining coat may be removed by gently applying pressure on a cover slip and sliding the cover slip back and forth. The tips of mature

asci are weakly stained in Congo red. A hyaline ring is barely visible at the apex.

Ultrastructurally, the apical wall of the diploid ascus (Fig. 3) is distinguishable in form from the rest of the ascal wall. Lomasomal activity occurs throughout the upper portion of the ascus. A thin, uneven, mucilaginous coat, 135-160 nm thick, is also present in the immediate area of the tip. The wall at this stage in development has a thickness of 135-170 nm.

By the early stage of ascospore delimitation, the mucilaginous coat has expanded notably both in thickness, being 375-415 nm at the tip, and in length, extending 6.5-7.5  $\mu\text{m}$  down the lateral face of the ascus (Fig. 4). The apical wall remains thin, being 140-170 nm thick, while the lateral wall has increased to 380-420 nm.

By late ascosporogenesis, the apical wall has broadened conspicuously (Fig. 5). At the same time, an annular indentation is formed delimiting the operculum. When thin sections are treated with silver methenamine, the layering of the ascal wall becomes sharply demarcated (Fig. 6). The increased thickening of the tip, which measures 310-360 nm, results from the addition of an inner layer, 250-280 nm thick. The adjacent suboperculum has a length of 6.3-7.1  $\mu\text{m}$ . The strongly stained inner layer narrows from 190-210 nm in thickness near the annular indentation to 75-85 nm at the base of the suboperculum. Conversely, the outer layer expands from 90-100 nm to 280-340 nm. Overall, the lateral

ascal wall narrows approximately 0.1  $\mu\text{m}$  as it approaches the operculum.

At ascospore maturity, the annular indentation is more defined (Figs. 7, 8) due to additional thickening of the inner layer of the operculum. This narrowed ring (Figs. 9, 10), having a length of 620-650 nm and thickness of 210-260 nm, consists of a greatly reduced inner layer, 130-170 nm, and a thin outer layer, 85-95 nm. Closer examination of sections stained with silver methenamine reveals the presence of a subtending, cytoplasmic ring (Fig. 9) which may play a critical role in ascospore discharge. The inner layer of the operculum has increased in thickness by an additional 20-45 nm (Fig. 8). Wall dimensions of the suboperculum remain essentially unchanged.

#### The Apical Apparatus of *Ascobolus crenulatus*

Diploid asci are fairly small and cylindrical, being 20-45  $\mu\text{m}$  long and 6-10  $\mu\text{m}$  wide. As the spores mature, the ascus grows to a length of 115-130  $\mu\text{m}$  and width of 12-15  $\mu\text{m}$ . When placed in IKI, blueing of the ascal wall is not detected. The tip, which appears thick-walled, has a slight conical shape and is bordered by a barely visible ring (Fig. 11). At maturation the tip becomes inflated and thinner-walled (Fig. 12).

Electron microscopic observations of the apex of the diploid ascus demonstrate the presence of localized vesiculation which is bounded by a ring of glycogen (Fig. 13). During early spore development, the apical wall, which has

become rounder in form, still consists of a single, uniform layer, 85-95 nm thick, toward the apex, and 100-110 nm thick in the lateral region (Fig. 14). Further on in spore maturation the apical wall undergoes a differential increase in thickness resulting in the formation of an annular indentation and an operculum (Fig. 15). With the aid of silver methenamine, the layering of the ascal wall is revealed. Throughout the operculum the outer layer has a thickness of 85-95 nm (Fig. 16). In contrast, the inner layer is less uniform in thickness, expanding from approximately 110-115 nm in the distal area of the operculum to 260-280 nm at its periphery. The operculum at this stage has a diameter of 4.4-4.5  $\mu\text{m}$ .

When the spores approach complete development, the width of the ascal tip increases from 5.9-6.3  $\mu\text{m}$  (Fig. 15) to 8.0-8.4  $\mu\text{m}$  (Figs. 17, 18). At the same time, the diameter of the operculum has widened to 5.7-5.9  $\mu\text{m}$ . The opercular wall layers, however, are reduced in thickness, implying a stretching action. The inner layer has decreased to 85-95 nm distally and 130-140 nm peripherally and the outer layer to 65-70 nm and 85-95 nm, respectively (Figs. 17, 18).

The annular indentation has a width of 545-570 nm, being smaller than in Peziza succosa (Fig. 19). The outer layer tapers slightly to 60-75 nm while the inner layer narrows to 35-40 nm. At the distal region of the suboperculum, (Figs. 18, 19), the breadth of the outer layer suddenly increases to 110-120 nm and maintains this approximate

thickness throughout the rest of the suboperculum. The inner layer, which is 40-50 nm thick for a length of 680-720 nm, expands to 100-120 nm at the lower end of the suboperculum. The suboperculum is roughly 3.5-4.0  $\mu\text{m}$  long.

#### The Apical Apparatus of *Saccobolus depauperatus*

The mature ascus is broadly clavate, being 55-80  $\mu\text{m}$  long and 12-15  $\mu\text{m}$  wide. When placed in IKI, the entire ascus stains blue. The apical apparatus is not observed until late ascosporogenesis whereupon a thick ring is detected at the tip (Fig. 20). At a slightly later stage in development the tip appears more inflated and less conspicuously thick walled (Fig. 21).

Ultrathin sections of asci during early spore wall formation show that additional development of the ascal wall is initially restricted to the apex or to that area which will become the operculum (Fig. 22). Subsequently, the inner layer making up the rest of the ascus wall is laid down. A distinct mucilaginous coat, 65-90 nm thick, covers the ascus.

At maturity the tip, which has become broadly truncate, possesses a distinct annular indentation (Figs. 23, 25). The annular indentation consists of a broader inner layer, 65-80 nm, and a thinner outer layer, 55-60 nm (Fig. 24). As in *Peziza succosa*, the mucilaginous coat is more prominent at this region of the ascal tip. A zone of dehiscence is present at the basal end of the annular indentation. The

width of the indented ring is 545-600 nm, being almost identical to that of Ascobolus crenulatus.

As before, posttreatment with silver methenamine accentuates the stratified character of the ascal wall (Figs. 25, 26). Within the operculum, the outer layer remains 60-70 nm thick. The inner layer, however, ranges from 65-75 nm apically to 180-190 nm subapically. The diameter of the operculum measures 7.1-7.4  $\mu\text{m}$ . In the apical portion of the suboperculum, the outer layer is less reactive with the silver methenamine stain (Fig. 25). This differentially stained region, 1.2-1.3  $\mu\text{m}$  long, will be referred to as the subopercular flange (Figs. 26, 28). The inner layer has a breadth of 65-85 nm throughout the suboperculum, which is 3.3-3.9  $\mu\text{m}$  long. The outer layer is 155-165 nm thick in the subopercular flange and decreases slightly to 140-145 nm before it once more increases to 220-230 nm in the lower end of the suboperculum. At incipient spore release, the operculum begins to pull away from the suboperculum along the annular indentation (Fig. 27). After the eight ascospores have been discharged as a solid unit, the flange extends outward and shrinks to 100-150 nm in length (Fig. 28).

The Apical Apparatus of Thecotheus pelletieri

Thecotheus pelletieri is the only representative of the 5 species examined that forms 32-spored asci. The mature ascus is broadly cylindric, having a length of 300-330  $\mu\text{m}$  and a width of 50-55  $\mu\text{m}$  (Fig. 29). The ascal wall becomes diffusely blue when treated with Melzer's reagent. The



apical apparatus consists of a large, conical operculum that is subtended by a wide ring (Fig. 30). In Congo red, the operculum appears to be distinctly thinner-walled than the rest of the ascus. Furthermore, the wall below the apical ring appears to be bilayered with the outer layer being stained by the Congo red (Fig. 30). At dehiscence of the ascus, the lid is often removed entirely (Fig. 31).

With the aid of the electron microscope, the form of the ascal tip is clearly shown (Fig. 32). The wide ring is seen to be an annular indentation, similar to that in the three previous species, but here of considerable size, measuring 980-1200 nm across. The operculum has a diameter of 21-23  $\mu\text{m}$  and a thickness that varies from 610-640 nm in the apical region to 720-750 nm subapically.

When stained with silver methenamine (Figs. 33, 34), the less reactive outer layer is seen to constitute much of the opercular wall, being 350-440 nm thick. In the area of the suboperculum, the outer layer increases basally from 510-590 nm to 1,150-1,250 nm. Similarly, the subopercular inner layer increases to 600-650 nm toward the base. The length of the suboperculum is 6.5-7.5  $\mu\text{m}$  long. A thin mucilaginous coat, being 80-100 nm thick, covers the entire ascal wall (Fig. 34). Both layers of the ascus are notably thinner in the annular indentation with the inner layer being reduced in thickness to 200-220 nm and the outer layer to 165-195 nm (Figs. 34, 35). Furthermore, a zone of dehiscence is observed at the lower end of the indented inner layer.



The Apical Apparatus of *Iodophanus granulipolaris*

The diploid ascus is broadly cylindrical, being 100-140  $\mu\text{m}$  long and 15-20  $\mu\text{m}$  in diameter. When placed in Melzer's Reagent, the staining reaction of the wall is partially masked by the coloration of the cytoplasm within (Fig. 36). The area immediately below the ascus tip turns to a light blue. Most of the ascus appears light green except centrally where it also stains blue. One-micron sections stained with toluidine blue (Fig. 37) shows the presence of densely packed cytoplasm in the apical region of the ascus. The apical region is bounded by a large, amorphous cylinder of deeply stained material. This cylinder extends to the center of the ascus where the large diploid nucleus is observed surrounded by cytoplasm. The remainder of the ascus is filled with another deeply staining mass of material.

The mature ascus, which retains a broadly cylindric form, expands to 200-250  $\mu\text{m}$  in length and 30-35  $\mu\text{m}$  in width. The wall stains a light blue when treated with the IKI solution. This is most clearly observed in empty asci. The apices of mature asci lack conspicuous features (Fig. 43). During ascospore liberation the operculum remains partially attached to one side of the ascus (Fig. 44).

Ultrastructurally, the tips of diploid asci appear to be cytologically active, being packed with ribosomes, endoplasmic reticula and mitochondria (Fig. 38). The large, amorphous cylinder consists of a uniform mass of glycogen

(Figs. 38, 40). Plasmalemmasomes are associated frequently with the apical wall and occasionally with the lateral wall (Figs. 39, 40). The apical wall, being 155-165 nm thick, is slightly thinner than the lateral wall, which is 200-210 nm.

By early ascosporeogenesis, the ascal tip becomes highly vacuolated as the central cylinder of glycogen appears to be degraded (Fig. 41). Silver methenamine stains only the distal region of the tip where the wall is 160-175 nm thick (Fig. 42). Basally, the wall thickens to 240-265 nm.

As the ascospores continue to develop, the apical wall changes little in form and thickness (Figs. 45, 46). The lateral walls, however, broaden to 420-460 nm. The apical region is filled with numerous, small vesicles.

At maturity, the ascal tip reaches a thickness of 440-480 nm (Fig. 48). An apical apparatus is not apparent until the sections have been treated with silver methenamine (Fig. 47). The operculum consists of a thinner, richly stained, outer layer, 170-200 nm thick, and a broader, less reactive, inner layer, 270-290 nm thick. The zone of dehiscence is demarcated by the differential staining of the inner layer in a small, swollen ring. The inner and outer layers immediately below the dehiscent ring are apparently stretched, measuring 20-40 nm and 400-420 nm in thickness, respectively. At the lower end of the suboperculum, which is 2.5-3.0  $\mu\text{m}$  long, the breadth of the inner layer has increased to 70-80 nm while the outer layer retains the same

dimensions. During spore release, the operculum usually remains attached to the suboperculum, not breaking entirely from the outer layer (Fig. 49). The inner layer in the distal region of the suboperculum has thickened to 70-80 nm (Fig. 50).

### Discussion

Examination in the present study of the apical apparatus of five species of the operculate Discomycetes demonstrated for the most part homogeneity in form and uniformity in development. Light microscopic observations showed that in each representative, except Iodophanus granulipolaris, the mature ascal tips displayed certain distinctive features. These findings are in close agreement with those of van Brummelen (1967) who stated that in the genus Ascobolus the opercula have characteristic shapes depending on the taxon in question and Kimbrough (1969), who reported similar observations in Thecotheus.

Within the operculate Discomycetes, Kimbrough (1966a), Kimbrough and Korf (1967) and van Brummelen (1967) reported a bilayered condition for the unitunicate ascus. With the light microscope, this aspect was only sufficiently determined in Thecotheus pelletieri. In each case, including this study, the representatives studied formed large, thick-walled asci, which permitted this observation. Electron microscopic examinations of the ascal wall for each of the five species currently studied demonstrated a double layered wall.

The ontogenies of the apical apparatuses found in Peziza succosa, Ascobolus crenulatus, Saccobolus depauperatus and I. granulipolaris followed in general a three-stage sequence. First, wall synthesis in diploid asci occurred throughout all regions of the cell. Synthesis appeared to be most concentrated at or near the apex where lomasomes (plasmalemmasomes as defined by Heath and Greenwood, 1970) were noted most frequently. Second, during early ascosporeogenesis, the development of the ascal walls in each species was restricted mainly to lateral or subapical regions. This was conspicuously apparent in P. succosa and I. granulipolaris where the lateral walls became two or three times thicker than the apical walls. Third, during ascospore wall formation, the additional layering of the ascal wall was differentially deposited, starting at the apex and progressing downward. None of the representatives displayed any evidence supportive of Chadeffaud's (1942) conclusion that complete differentiation of the apical apparatus occurred by the eight-nucleated stage. Instead, the ontogeny of the apical apparatus of each species showed the most dramatic change during late ascosporeogenesis. Observations made in the present study coincided closely with those of van Brummelen (1967) and Wells (1972) regarding the time of opercular development in Ascobolus species.

In the young asci of A. crenulatus and maturing asci of I. granulipolaris, the localized accumulation of vesicles below the tip was reminiscent of the vesicle system described

for the developing diploid ascus of Xylaria longipes Nitschke (Beckett and Crawford, 1973; Beckett, Heath and McLaughlin, 1974) and the growing hyphal tips of various taxa throughout the fungal world (Grove and Bracker, 1970). Unlike in X. longipes, an apical body (Spitzenkörper) and an area of localized thickening were not observed during these stages of development in the species studied by me. Wells (1972) observed a similar state of vacuolation in Ascobolus stercorarius prior to meiosis and noted that it was part of an orderly sequence. He suggested that the vacuoles may function in concentrating the ascoplasm in the regions of growth and cytological activity.

The gross morphology of the apical apparatuses was basically similar in all members. Except in Thecotheus pelletieri, the inner layer was seen to be distinctly broadest in the opercular region of the ascus. In every species, the outer layer increased in thickness towards the base of the ascus. These findings compared favorably with those reported for the six representatives of the Sarcoscyphineae (Samuelson, 1975). However, they sharply contrasted with Schrantz' (1970) description of Peziza plebeia. Schrantz defined the outer ascal layer as consisting of a "pectic" external cover and an "amyloid" inner hood or "manchon" (Fig. 5lb). His light and electron microscopic descriptions of the outer layer are nearly identical in size and shape to the mucilaginous coat of Peziza succosa (Fig. 5ld). His photomicrograph of the ascal tip of P. plebeia showed a

comparable stage in development to that of the tip seen in Fig. 4 for P. succosa. He apparently based his evidence on immature asci.

A substructural characteristic found in the apical apparatus of all species, except I. granulipolaris, was the presence of an annular indentation. Previously, Kimbrough (1966b, 1969) reported the occurrence of a "well-marked indentation at the operculum" in mature asci of Thecotheus pelletrieri and of less obvious lines of dehiscence in the eight-spored species of Thecotheus. Van Brummelen (1967) did not specifically describe this region in any species of Ascobolous and Saccobolus. Instead, he stated that the annulus, an internal ring-shaped thickening of the ascal wall, was the place where circumscissile rendering of the lid had occurred. Chadeffaud (1942) had depicted earlier (Fig. 51a) an open area between the operculum and the subapical "bourrelet" or pad. Although he did not name the area or refer to it in particular, he mentioned that the operculum was unusually well defined. In a recent description of the development of the ascal wall in Ascobolus stercorarius, Wells (1972) noted that the differentiation of the operculum began "with the appearance of an annular indentation in the inner surface of the wall at the margin of the operculum." His accounts of the development of the lateral and apical walls and his measurements of their breadth and width were very similar and in some cases identical to my observations of A. crenulatus (Fig. 51c).



The discovery of an annular indentation in Peziza succosa was not entirely unexpected. Kimbrough had noted frequently the presence of a distinct hyaline ring in the ascus tips of P. vesiculosa when stained with Congo red (personal communication). My observations of P. succosa were similar though the staining reaction appeared less pronounced. We had conjectured that the ring may have been the result of either a chemical differentiation in the wall or a physical thinning of the wall. The latter proved to be true.

Of the representatives that had annular indentations, the species which turned blue in Melzer's reagent, i.e., P. succosa, S. depauperatus and T. pelletieri, possessed mucilaginous coats. It is believed that the iodine specifically reacted with the mucilaginous coats. Asci of P. succosa provided the most convincing evidence. The localization of the blueing reaction in young and mature asci coincided perfectly with the localization of their respective mucilaginous coats. Moreover, the coat could be removed after delicate manipulation. In T. pelletieri and S. depauperatus, the total blueing of the ascus walls corresponded entirely with the thin mucilaginous layer that covered the ascus. Although asci of A. crenulatus did not exhibit mucilaginous coats, neither did they give an iodine-positive reaction in Melzer's reagent.

The apical apparatus of P. succosa seemed the most isolated morphologically of all the species studied that developed annular indentations. Major differences consisted



of the presence of a thick, localized mucilaginous coat, a subtending cytoplasmic ring and an operculum which thickened distally rather than thinned (Fig. 5ld). In addition, the inner layer of the suboperculum was seen to taper notably in thickness away from the tip. By comparison, the apical apparatuses of A. crenulatus and S. depauperatus shared a number of identical features, including the width and breadth of their annular indentations, opercular and subopercular flanges. The principal difference between the two members was observed in the layering of the lower suboperculum. The expansion of the inner layer toward the base of the ascus in A. crenulatus in Fig. 5lc (vs. S. depauperatus) was similar to the suboperculum of T. pelletieri. The apical apparatus of T. pelletieri was generally similar in form to that of A. crenulatus. For the most part, the ascal wall dimensions of T. pelletieri were approximately three times that of A. crenulatus.

The apical apparatus of Iodophanus granulipolaris diverged significantly in form from the other species and also in development and cytochemistry. The presence of a ring of dehiscence in place of an annular indentation was the chief morphological difference. Furthermore, the differential staining of the inner layer along the zone of dehiscence by silver methenamine was dissimilar to the rest of the species. The suboperculum was considerably shorter and less conspicuous than those of the annular indented species. During the ontogeny of the apical apparatus, the

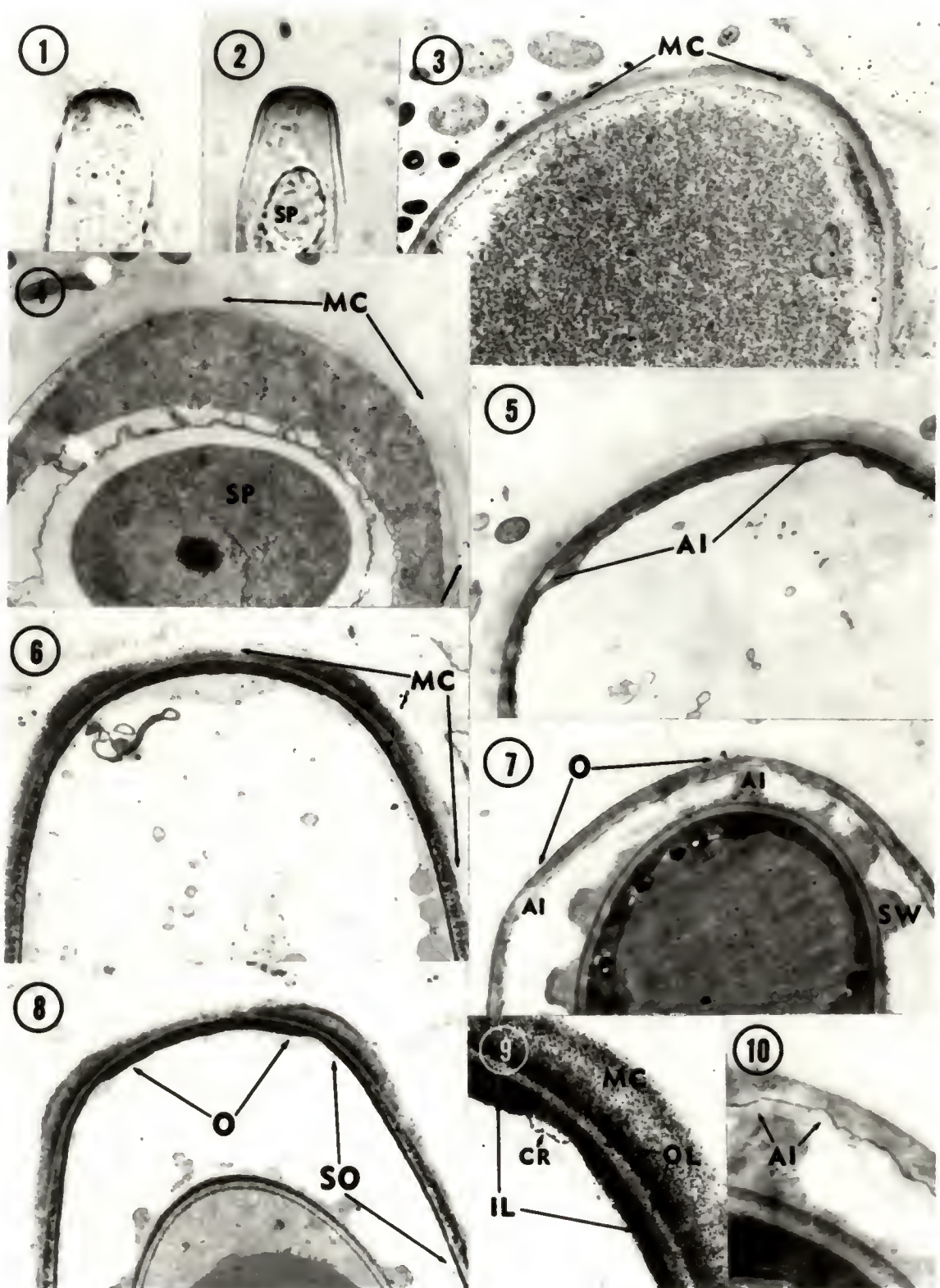
expansion of the apical wall was later and more restricted in locality than observed for the other species. Since the presence of an exogenous coat was not detected at any stage of ascal development, the iodine-positive reaction appeared to have been the result of iodine directly staining the wall.

The iodine-positive apical apparatus has been demonstrated to contain distinct features that can be useful as a systematic tool. The marked similarities in form and development in the dehiscent structures of A. crenulatus and S. depauperatus further substantiated the close association of the two genera. The morphology of the apical apparatus of T. pelletieri and its resemblance to Ascobolus supported Korf's (1973) taxonomic treatment of this genus, placing it in the Ascobolaceae. Although the apical apparatus of P. succosa differed significantly from the other annular indented taxa, the number of common properties shared by the four species indicated a closer degree of relatedness than was proposed previously by Chadeaud (1942) and Eckblad (1968). Except in gross form and ontogeny, the apical apparatus of I. granulipolaris had very few properties in common with those of the annular indented species. The taxonomic positioning of I. granulipolaris in either the Pezizaceae or the Ascobolaceae received little support from the present study. Since blueing in iodine may be the result of more than one site of activity, the iodine-positive character should be interpreted cautiously.

## Chapter I

### Figures 1-10. Peziza succosa

- Figure 1. Tip of diploid ascus showing iodine-positive reaction. Stained with Melzer's reagent. X1,000.
- Figure 2. Diffuse iodine-positive reaction at mature ascal tip. Spore (SP). X1,000.
- Figure 3. Thin mucilaginous coat (MC) covers immediate region of the apex of diploid ascus. X10,000.
- Figure 4. Ascal tip with thick mucilaginous coat (MC) at early spore delimitation. Spore (SP). X7,800.
- Figure 5. Presence of annular indentation (AI) by late ascosporogenesis. X9,000.
- Figure 6. Ascal tip at late ascosporogenesis. Mucilaginous coat (MC). Stained with silver methenamine. X6,000.
- Figure 7. Mature apical apparatus. Distinct annular indentation (AI) delimits operculum (O). Spore wall (SW). X7,600.
- Figure 8. Opercular (O) and subopercular (SO) regions of mature apical apparatus. Stained with silver methenamine. X7,000.
- Figure 9. Close-up of Fig. 8 showing cytoplasmic ring (CR) which subtends annular indentation. Inner layer (IL). Outer layer (OL). Mucilaginous coat (MC). Stained with silver methenamine. X20,000.
- Figure 10. Region of dehiscent zone which is demarcated by annular indentation (AI). X24,000.

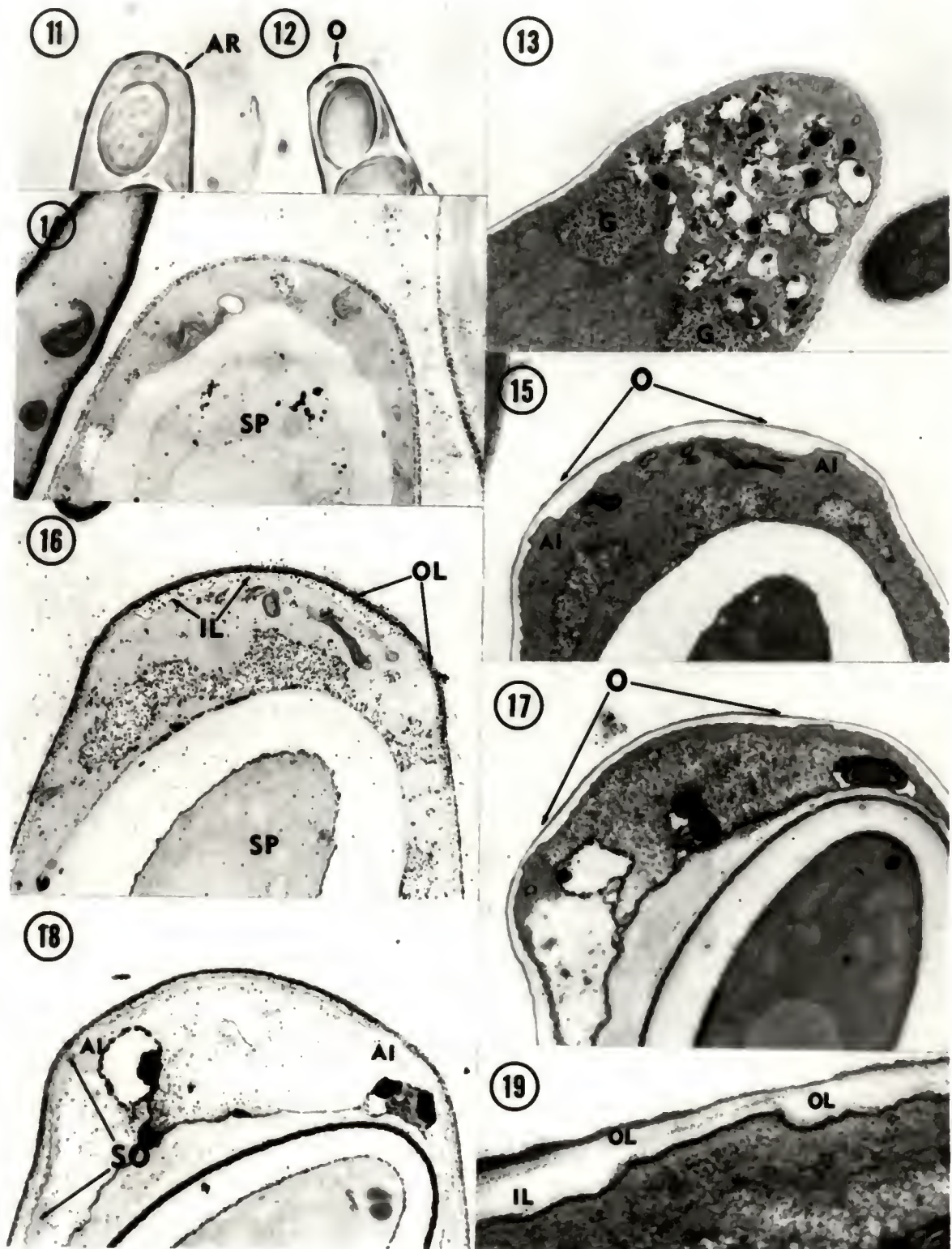


## Chapter I

### Figures 11-19. Ascobolus crenulatus

- Figure 11. Young ascal tip with developing ascospores shows distinct apical ring (AR) at this time. Stained with Congo red. X1,250.
- Figure 12. Mature tip is thinner-walled at region of the operculum (O). Stained with Congo red. X1,000.
- Figure 13. Vesiculated apex of diploid ascus with subtending ring of glycogen (G). X9,800.
- Figure 14. Ascal tip during early spore (SP) development. Stained with silver methenamine. X10,500.
- Figure 15. Early formation of apical apparatus showing differential increase in thickness of opercular (O) wall. Annular indentation (AI). X11,000.
- Figure 16. Inner layer (IL) and outer layer (OL) of developing apical apparatus. Spore (SP). Stained with silver methenamine. X10,500.
- Figure 17. Mature apical apparatus with umbonately shaped operculum (O). X9,500.
- Figure 18. Mature apical apparatus shows layering of opercular and subopercular (SO) regions of the ascus. Annular indentation (AI). Stained with silver methenamine. X8,500.
- Figure 19. Region of annular indentation in mature apical apparatus. Inner layer (IL). Outer layer (OL). X48,000.



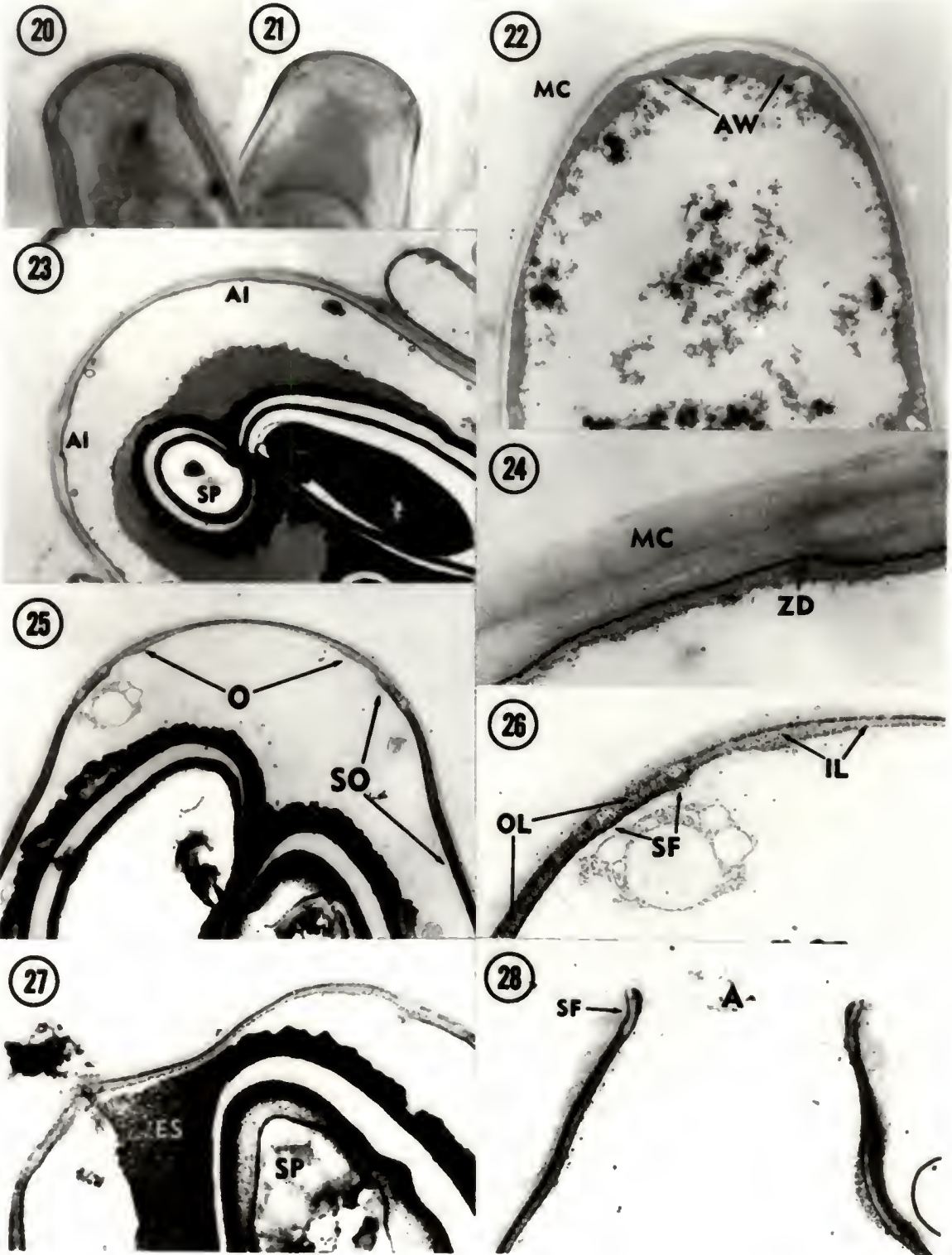


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- Figure 28. Dehiscent ascus with outwardly extended subopercular flange (SF). Ascostome (A). Stained with silver methenamine. X6,200.

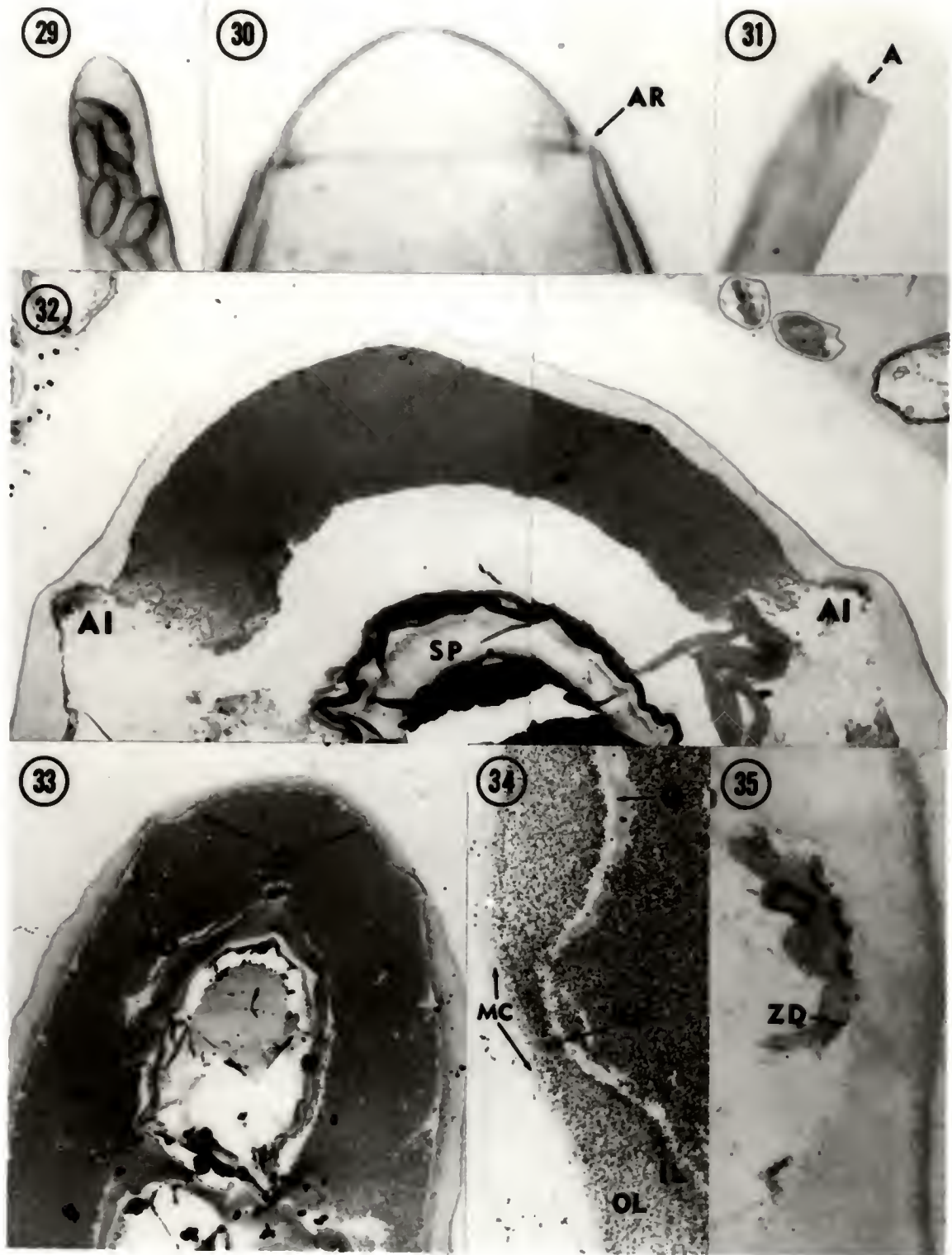




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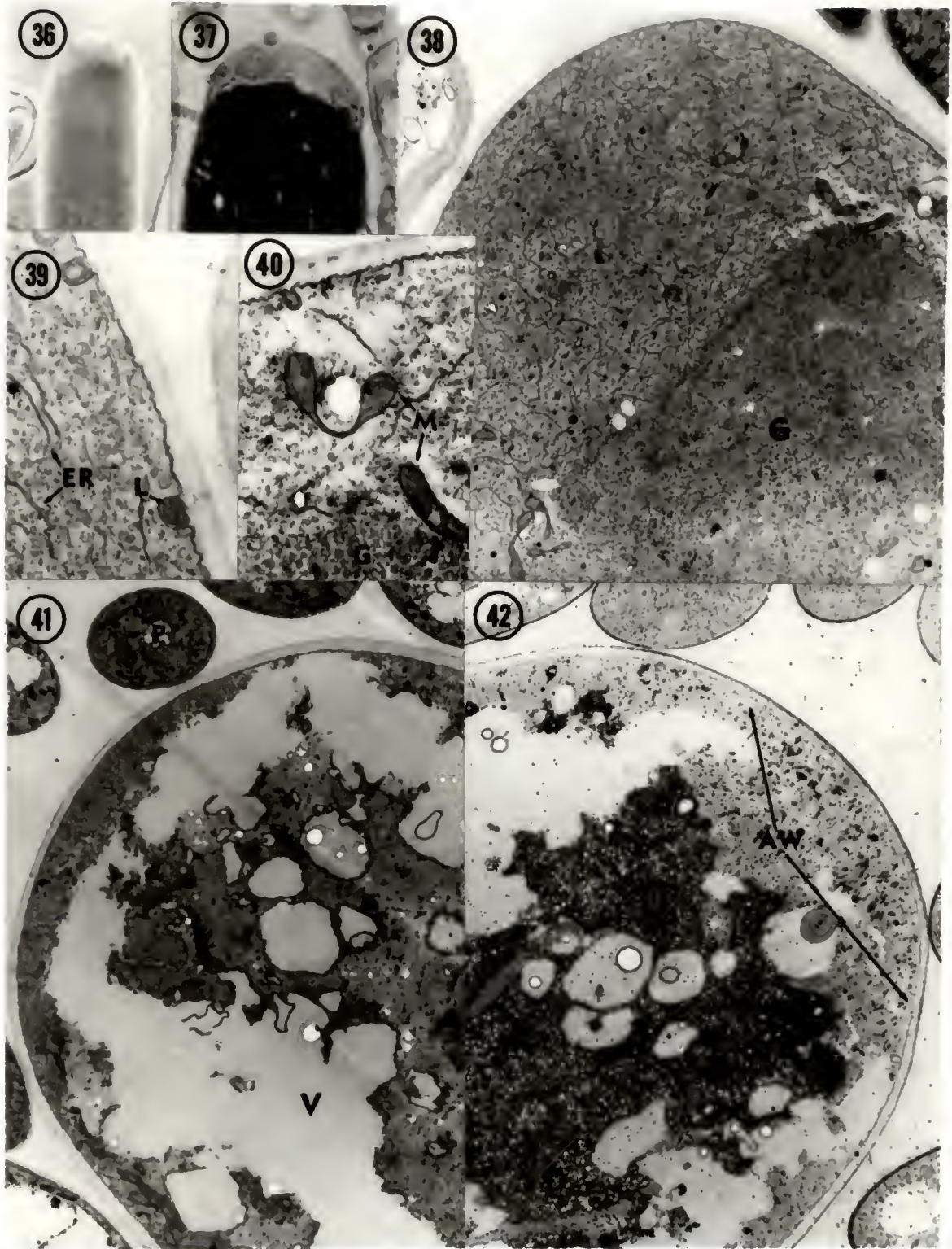


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- Figure 37. Apex of uninucleated ascus subtended by broad amorphous cylinder. One-micron section stained with toluidine blue. X1,000.
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- Figure 39. Small lomasomes (L) at ascal tip. Endoplasmic reticulum (ER). X20,000.
- Figure 40. Mitochondria (M) adjacent to large mass of glycogen (G). X15,000.
- Figure 41. Large vacuole (V) at tip during early ascosporeogenesis. X4,700.
- Figure 42. Distal portion of apical wall (AW) stained with silver methenamine. X4,700.



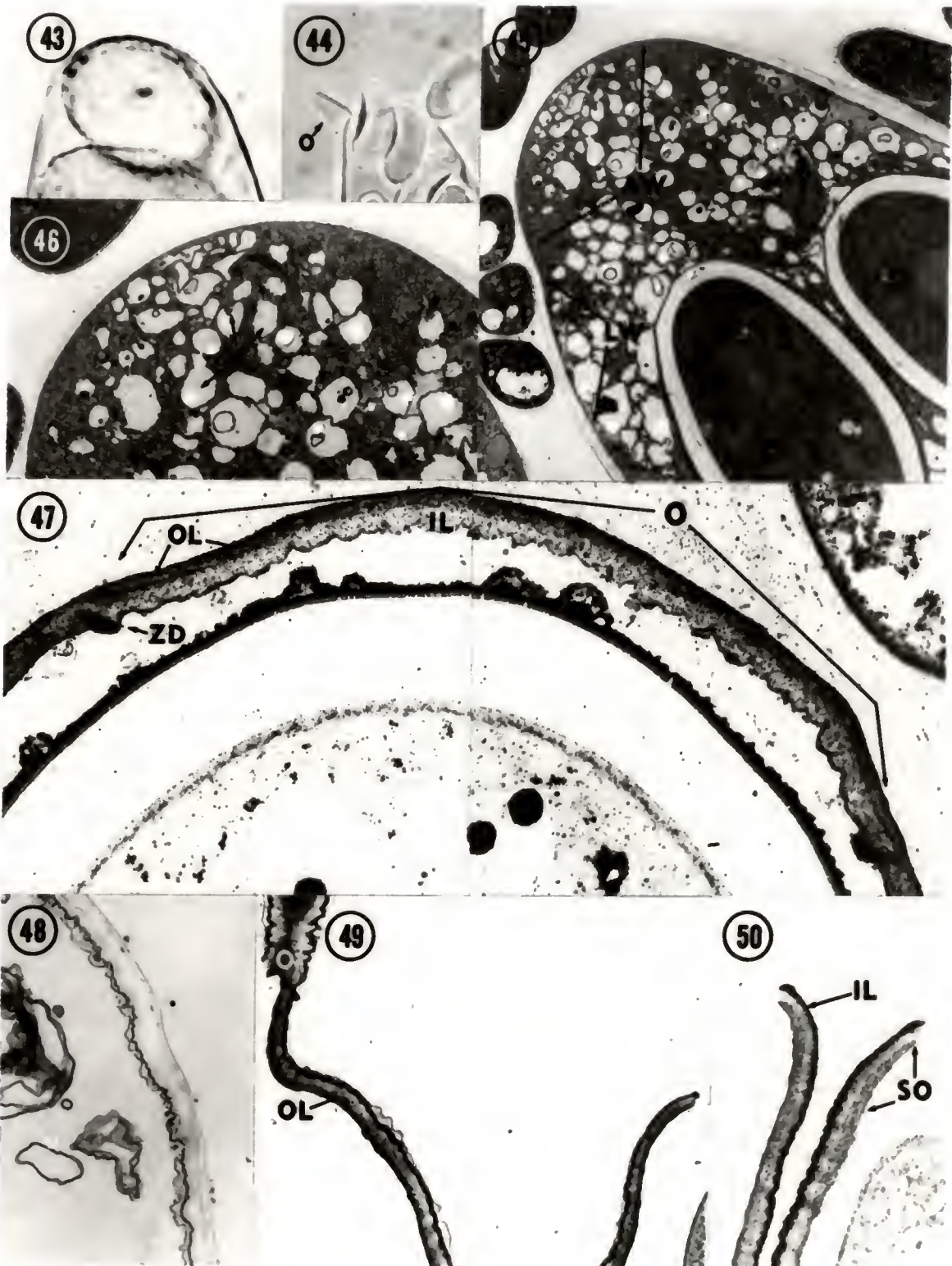


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### Figures 43-50. Iodophanus granulipolaris

- Figure 43. Mature tip stained with Congo red. X1,250.
- Figure 44. Operculum (O) partially connected to ascus at spore liberation. Stained with Melzer's reagent. X400.
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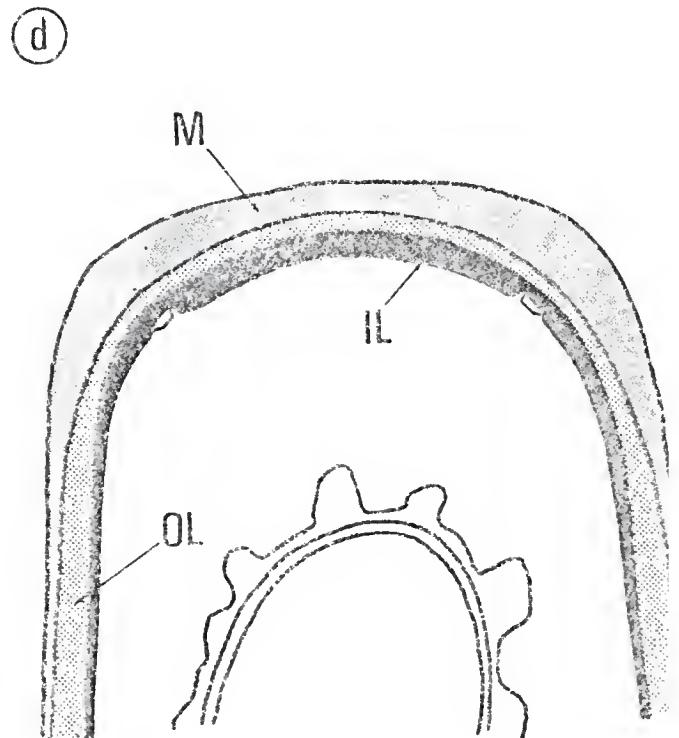
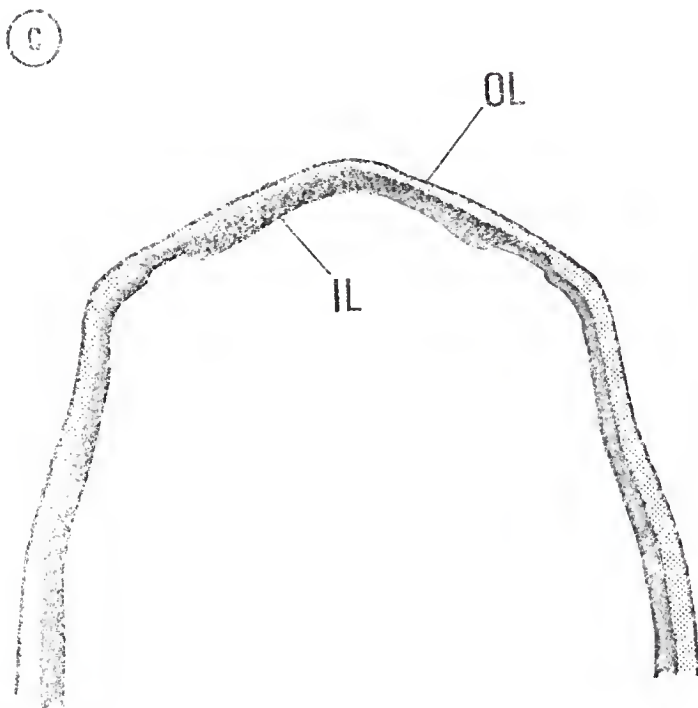
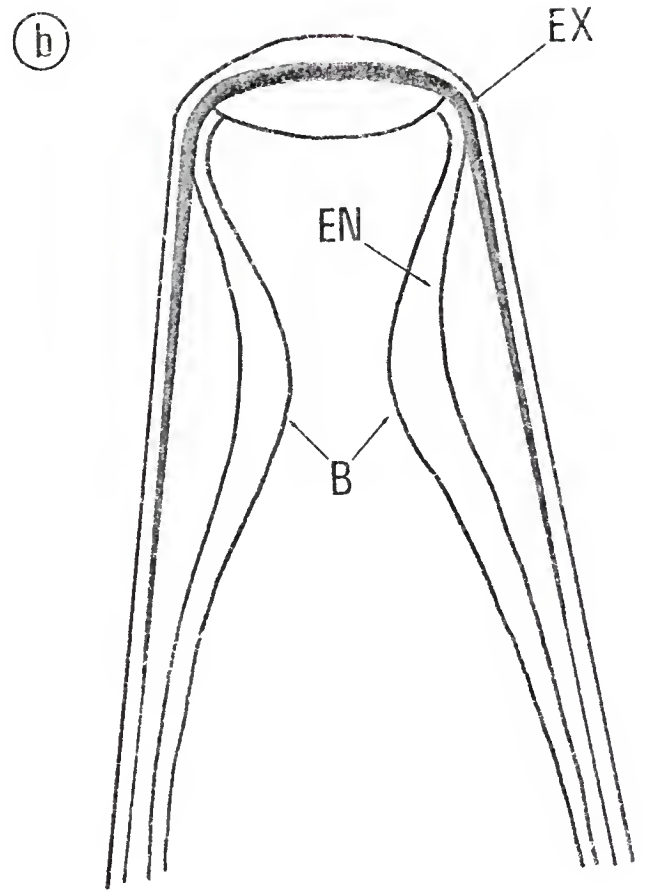
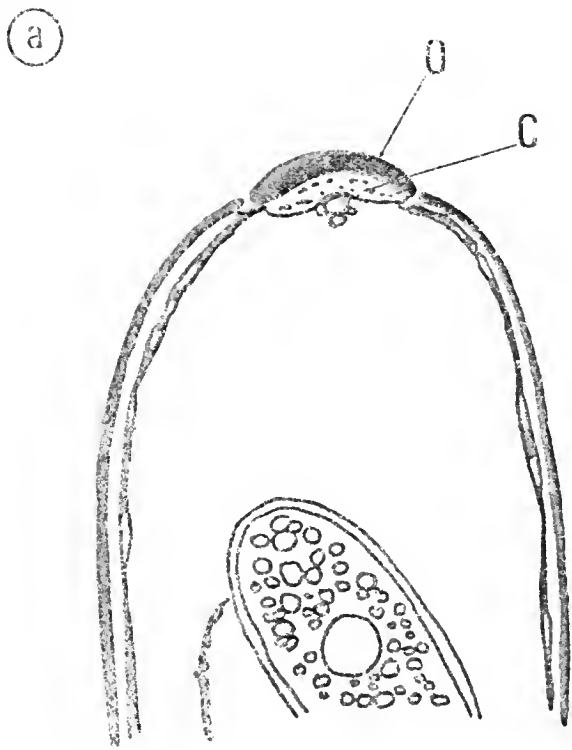




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Figure 51. Drawings of apical apparatuses found in iodine-positive asci.

- a. Apical apparatus of Ascobolus furfuraceus. Operculum (O). Coussinet (C). Redrawn from Chadeffaud (1942).
- b. Ascal tip of Peziza plebeia illustrating layers of the wall. Exoascus (EX). Endoascus (EN). Bourrelet (B). Redrawn from Schrantz (1970).
- c. Illustration made from electron microscopic observations of the wall layering in mature ascal tips of Ascobolus crenulatus. Outer layer (OL). Inner layer (IL).
- d. Illustration made from electron microscopic observations of the wall layers and mucilaginous coat (M) in mature ascal tips of Peziza succosa. Outer Layer (OL). Inner layer (IL).



CHAPTER II  
MORPHOLOGY, DEVELOPMENT AND CYTOCHEMISTRY OF THE APICAL  
APPARATUSES OF REPRESENTATIVES IN THE OTIDEA-ALEURIA COMPLEX

Introduction

In the area of fungal systematics, searches have long been made for valid, new characters which would aid in classification. With the incorporation of cytological information developed by Berthet (1964) and pigmentation studies made by Arpin (1968) there has surfaced a general taxonomic repositioning of the genera representing the largest group of operculate Discomycetes (Table 1). These members have been treated successively during the last decade in the Humariaceae and Pezizaceae, tribe Otideae (Dennis, 1968), Humariaceae (Rifai, 1968), Pyronemaceae and Otideaceae (Eckblad, 1968), Aleuriaceae and Otidiaceae (Kimbrough, 1970), and Pyronemataceae (Korf, 1973). Circumscription of groupings of these taxa, which will be referred to as the Otidea-Aleuria complex, traditionally has been difficult due to the occurrence of continuous patterns of variation in several characters.

Within the Ascomycetes increasing emphasis has been placed on ascal structure and the mechanism of ascospore release (see General Introduction). Chadeaud (1942, 1973), the first worker to examine the morphology of ascal tips

Chapter II  
Table 1

Classifications of the Otidea-Aleuria Complex

Dennis (1968)

Eckblad (1968)

Humariaceae

Pyronemaceae

Lachneae

Sepultaria  
Leucoscypha  
Tricharia  
Humaria  
Trichophaea  
Pseudoomphilia

Iodophanus  
Pyronema  
Pyronemella  
Lamprospora  
Pulvinula  
Caloscypha  
Octospora  
Leucoscypha  
Coprobria

Ciliareae

Scutellinia  
Cheilymenia  
Sphaerosporella  
Neottiella  
Melastiza  
Anthracobia

Cheilymenia  
Scutellinia  
Humaria  
Tricharia  
Sphaerosporella  
Sepultaria  
Jafnea  
Fimaria  
Pseudombrophila  
Anthracobia  
Melastiza  
Aleuria

Aleurieae

Aleuria  
Pulvinula  
Coprobria  
Geopyxis  
Fimaria  
Octospora  
Psilpezia  
Boudiera  
Caloscypha  
Lamprospora

Otideaceae

Geopyxis  
Pustulina  
Sowerbyella  
Otidea  
Ascosparassis

Pezizaceae

Rifai (1968)

Otideae

Humariaceae

Otidea  
Barlacina  
Pseudotis  
Sowerbyella  
Pustularia

Otideae

Otideae  
Marcelleina

Lachneae



Table 1 (Cont'd.)

## Humariaceae

Jafnea  
Jafneadelfus  
Nothojafnea  
Sphaerosporella

## Ciliarieae

Rhizoblepharia  
Scutellinia  
Cheilymenia  
Coprobia

## Aleuriaceae

Anthracobia  
Melastiza  
Aleuria  
Leucoscypha  
Geopyxis  
Octospora  
Inermisia  
Pulvinula  
Lamprospora

Kimbrough (1970)

## Otidiaceae

Otidea  
Pustulina  
Jafnea  
Nothojafnea  
Jafneadelfus  
Sowerbyella  
Ascosparassis  
Sepultaria  
Tricharia  
Mycolachnea  
Pseudotis  
Barlaeina  
Pseudombrophila  
Trichophaea

## Aleuriaceae

Coprobia  
Cheilymenia

## Aleuriaceae

Scutellinia  
Geopyxis  
Aleuria  
Melastiza  
Octospora  
Anthracobia  
Caloscypha  
Sowerbyella  
Fimaria  
Leucoscypha  
Inermisia  
Genosperma  
Rhizoblepharis

Korf (1973)

## Pyronemataceae

## Ascodesmidoideae

Ascodesmis  
Sphaerozone  
Pulparia  
Jafneadelfus

## Pyronematoideae

## Pyronemateae

Pyronema

## Karstenelleae

Karstenella

## Ascophanoideae

## Geopyxideae

Geopyxis  
Apapaphysaria

## Pseudombrophileae

Selenaspora  
Tricharina  
Rhizoblepharina



Table 1 (Cont'd.)

## Pyronemataceae

Trichophaeopsis  
Pseudombrophila  
Fimaria

## Otideoideae

## Jafneae

Jafnea  
Tarzetta

## Otieae

Psilopezia  
Otidea  
Ascosparassis

## Mycolachneae

Nothojafneae  
Geopora  
Humaria  
Trichophaea

## Scutellinioideae

## Scutellinieae

Coprobia  
Scutellinia  
Cheilymenia

## Sowerbyelleae

Sowerbyella  
Caloscypha  
Acervus  
Phaedropezia

## Pyronemataceae

## Aleurieae

Anthracobia  
Hiemsia  
Melastiza  
Leucoscypha  
Aleuria  
Pseudocollema  
Pulvinula  
Lamprospora  
Octospora  
Byssonectria  
Kotlabaea

throughout the Euascomycetes, concluded that ascal apices, in general, shared a number of common features and that the absence or presence of certain features had phylogenetic significance. Recent ultrastructural investigations of the operculate ascus (van Brummelen, 1974, 1975; Samuelson, 1975; Schrantz, 1970; Wells, 1972) and that reported in Chapter I have demonstrated that the ascal tip has a broad variability in form. Furthermore, comparative analysis of the morphology and ontogeny of apical apparatuses of different taxa may have considerable phylogenetic value as discussed in Chapter I, thus giving support to Chadeaud's conclusions.

Within the suborder Pezizineae, ascal structure has been little studied outside of the Thelebolaceae (Kimbrough, 1966a, 1966b, 1972; Kimbrough and Korf, 1967, van Brummelen, 1974) and Ascobolaceae (van Brummelen, 1967; Wells, 1972; Chapter I). Chadeaud's (1942) work represented the major comprehensive study of the apical apparatus for the remainder of the operculate Discomycetes. Of the 11 selected taxa described, 8 were members that fell into the Otidea-Aleuria complex. They included Aleuria aurantia (Pers. ex Hook.) Fuckel, Humaria hemisphaerica (Wigg. ex Fr.) Fuckel, (as Lachnea hemisphaerica), Coprobria granulata (Bull.) Boud., Pulvinula constellatio (Berk and Br.) Boud., Scutellinia hirta (Schwm. ex Fr.) O. Ktze., Octospora leucoloma Hedw. ex. S. F. Gray (as Humaria leucoloma), Humaria wrightii (Berk. and Cooke) Boud. and Sepultaria arenosa (Fuckel)

Boud. Each of the eight species was observed to have a full complement of apical structures associated with the operculate apical apparatus as shown in Fig. 110A. Four morphological variations of the apical apparatuses were demonstrated among the representatives. They ranged from species with exaggerated or exceptionally well-developed apical apparatuses as in C. granulata to those that had rudimentary forms as in O. leucoloma (Fig. 110D). Chadeffaud proposed that the differences in their morphology were the result of regressive evolution and that in the case of the distantly related genus Ascobolus, all species had attained a similarly reduced state by this means.

Eckblad (1968) confirmed the presence of a funnel in a number of unstated species and agreed with Chadeffaud's description of the operculate apical apparatus on all main points. He stated, however, that the funnel was cytoplasmic in origin and did not attach to the operculum. He also was not able to detect an apical pad or cushion on the underside of the operculum. Recent investigations of operculate apical apparatuses (Wells, 1972; Samuelson, 1975; van Brummelen, 1975; Chapter I) have demonstrated the presence of a thickened, opercular inner layer which corresponds with the apical pad. The apical globule, punctuation, funnel and tract were not described in any of the "iodine-positive" and suboperculate apical apparatuses.

LeGal (1953) described the ascal apices of Phaedropezia epispartia (Berk and Br.) LeGal and Trichophaea

erinaceus (Schwein.) LeGal, both placed inside the Otidea-Aleuria complex, to have apical apparatuses typical but less apparent than that of the suboperculates. Eckblad (1968) suggested that the occurrence of an incomplete ring in the suboperculate apical apparatus of T. erinaceus and P. epispartia as well as in those of the Sarcoscyphaceae were artifacts of fixation. He believed that the apical chamber, which contained the ring, represented only a swelling of the inner layer of the operculum. Samuelson's (1975) and van Brummelen's (1975) examinations of the suboperculate apical apparatuses corroborated Eckblad's observations. Nevertheless, LeGal's report of suboperculate apical apparatuses in T. erinaceus and P. epispartia indicated the possibility of pronounced wall layering of the ascal tips, a character that may prove to be taxonomically useful.

The only ultrastructural examination of the ascal wall found in the Otidea-Aleuria complex was made by Schrantz (1970) on Tarzetta cupularis (L. ex Fr.) Lamb. He noted that the exoascus or outer layer consisted of a thin, loosely woven sheath which thickened near the ascal tip. By comparison, the endoascus or inner layer was thick and finely granular and narrowed towards the tip. He likened the form of the ascal wall to that of Peziza plebeia (LeGal) Nannf. It was pointed out in Chapter I that the exoascus described for P. plebeia was apparently a thick mucilaginous coat. The ascal wall layering in T. cupularis may have been similarly misinterpreted.

This study has incorporated morphological, developmental and cytochemical examinations of the Otidea-Aleuria apical apparatus in order to (1) ascertain the validity of the features depicted by Chadeffaud, (2) compare the morphology and developmental sequences of apical apparatuses between selected representatives, (3) determine whether these features would be useful in the taxonomic positioning of members within this largest of all operculate groups. Ten members including Otidea leporina (Fr.) Fuckel, Sphaerosporella brunnea (Alb. and Schw. ex Fr.) Svrcek and Kub., Jafnea fusicarpa (Gerard) Korf, Humaria hemisphaerica, Aleuria aurantia, Anthracobia melaloma (Alb. and Schw. ex Fr.) Boud., Scutellinia scutellata (L. ex Fr.) Lamb., Ascozonus woolhopensis (Berk. and Br. apud Renny) E. C. Hansen, Sowerbyella imperialis (Peck) Korf, and Geopyxis majalis (Fr.) Sacc. were used.

### Materials and Methods

#### Collection and Age Determination of Material

Young and mature apothecia of Anthracobia melaloma were collected from charred wood near Gainesville, Florida. Different aged material of Humaria hemisphaerica was found growing in moss at the University of Florida's Horticultural Farm outside Gainesville. Young and mature apothecia of Aleuria aurantia and Jafnea fusicarpa and mature apothecia of Otidea leporina were found on the forest floor at the Devil's Millhopper in Gainesville. Young and mature

apothecia of Scutellinia scutellata were gathered from a greenhouse bench of the Department of Plant Pathology at the University of Florida. Fully developed apothecia of Sphaerosporella brunnea were found on carbonized humus at the Collier-Seminole State Park in Florida. Minute apothecia of Ascozonus woolhopensis were found on rodent dung collected in Macon County, North Carolina. Fresh material was brought to the laboratory where free-hand sections were made for light microscopic inspection to determine the stage of ascal development. Young and mature apothecia were studied separately when possible.

Dried specimens of Geopyxis majalis and Sowerbyella imperialis were obtained from the Mycological Herbarium at the University of Florida and Dr. R. P. Korf of Cornell University, respectively. Portions of apothecia were revived at room temperature in distilled water for 6 to 12 hours in a moist chamber for light and electron microscopic observations.

#### Procedures for Light Microscopic Examinations

Fresh and revived apothecia were cut into blocks, sectioned and mounted on slides as described in Chapter I. Congo red was predominantly used to stain the ascal walls (Samuelson, 1975). Aniline blue and lactophenol cotton blue were used to observe cytoplasmic detail (see Chapter I). Plastic embedded material was sectioned, mounted and stained in the manner described in Chapter I.



### Procedures for Electron Microscopic Examinations

Entire apothecia of A. melaloma, A. woolhopensis and S. scutellata and five millimeter squares of apothecia of A. aurantia, G. majalis, H. hemisphaerica, J. fusicarpa, S. imperialis and S. Brunnea were fixed in buffered (0.2 M sodium cacodylate pH 7.2) 2.0% glutaraldehyde and 2.0% paraformaldehyde solution for two hours at room temperature. Five millimeter squares of O. leporina and S. brunnea were fixed in 1.0% permanganate solution for one hour at room temperature. All materials were postfixed in osmium tetroxide, dehydrated, embedded, section and poststained as described in Chapter I.

### Results

Descriptions of the apical apparatuses for each species are restricted to the three regions of the mature ascal tip; the operculum, the zone of dehiscence and the suboperculum. As in the previous chapter each representative has been treated individually.

#### The Apical Apparatus of Otidea leporina

The mature ascus is narrowly cylindric, reaching a length of 180-200  $\mu\text{m}$  and a diameter of 9-12  $\mu\text{m}$ . The subapical region is notably heliotropic (Fig. 1). When stained in Congo red, the ascal wall appears thick for most of the length of the ascus. Near the tip of the ascus the wall becomes thinner below the region of the operculum (Fig. 2). By comparison, the opercular wall appears to be inflated.

The diameter of the ascus becomes narrower toward the tip. After the spores have been discharged, the ascus shrinks to a length of 150-170  $\mu\text{m}$  while its diameter expands to 10-15  $\mu\text{m}$  (Fig. 4). The operculum, which usually remains attached to one side (Fig. 5), is less conspicuous than when observed in undischarged asci.

Ultrastructurally, the apical wall protrudes above the subtending lateral wall, delimiting the operculum (Fig. 3). The operculum has a diameter of 2.2-2.4  $\mu\text{m}$  and a uniform thickness of 170-180 nm. The suboperculum is 3.7-4.0  $\mu\text{m}$  long and decreases in thickness from 510-530 nm at its lower extremity to 165-180 nm next to the operculum. The outer layer of the operculum and distal region of the suboperculum are loosely fibrillar and electron-dense, having a breadth of 110-120 nm (Figs. 3, 6). Toward the base, the outer layer thickens to 390-410 nm, having an additional internal portion or stratum. The inner layer and the internal stratum of the outer layer are both electron-transparent and granular, being separated by a faint, opaque band (Figs. 3, 9). After treating the thin sections with silver methenamine, the inner layer is stained most strongly (Fig. 7). The internal stratum of the outer layer is stained but not as intensely, and the electron-dense, fibrillar portion of the outer layer remains unstained. As the time of spore release approaches, the apical wall becomes markedly stretched (Fig. 8). The opercular diameter increases 0.3-0.5  $\mu\text{m}$  while its thickness decreases by 40-60 nm. The upper extremity of

the suboperculum is similarly stretched. At ascal dehiscence, the upper extremity of the suboperculum becomes outwardly extended (Fig. 9). The length of the suboperculum shrinks to 3.0-3.3  $\mu\text{m}$ .

#### The Apical Apparatus of *Jafnea fusicarpa*

During early spore formation, the ascal wall is thinner at the apex and thicker laterally (Fig. 10). Further in development, the apical and subapical walls are conspicuously thick (Fig. 11). At maturity, the ascus is long and narrow, 290-310  $\mu\text{m}$  x 18-22  $\mu\text{m}$ . The ascal tip, which is slightly heliotropic, has formed two layers (Fig. 13). The first spore often becomes lodged against the opercular region of the ascus. During spore release, the operculum is thrown to one side and the ascospores are ejected one after another in rapid succession. The operculum typically remains attached to the ascus and frequently returns to its original prone position. As the spores are released they become strongly compressed between the sides of the ascal wall (Fig. 14).

Electron microscopic examination of a four-nucleated ascus reveals for the most part the presence of a thick, lateral wall, 820-840 nm (Fig. 12), which decreases in thickness to 500-540 nm at the tip. Numerous small vesicles are observed in the apical region of the ascus. During early spore wall formation, the ascal tip becomes faintly heliotropic (Fig. 15). The thickness of the lateral wall has increased to 1000-1050 nm while the tip retains a thickness

of 480-520 nm. The apex is wider, having expanded to a diameter of 9.2-9.4  $\mu\text{m}$ . Several large vacuoles are present near the tip and extend to the base of the ascus. At the end of spore development the apical apparatus continues to be developed (Fig. 16). The ascus tip has increased by 1.3-1.8  $\mu\text{m}$  in diameter. An inner layer was deposited mainly in the vicinity of what will become the operculum. Lateral walls appear to be stretched, having a thickness of 780-820 nm. Treatment with silver methenamine strongly accentuates the bilayered nature of the mature ascus wall (Fig. 17). The thickness of the inner layer narrows from 400-410 nm at the operculum to 50-65 nm at the lower extremity of the suboperculum where a small annular bulge or ring has formed (Fig. 17, arrows). An opercular boundary is not observed at any time prior to spore release. At dehiscence, the operculum remains partially fastened to the suboperculum (Fig. 18). At this stage, the suboperculum, which is 5.5-6.3  $\mu\text{m}$  long, has thickened to 950-980 nm at its lower extremity where the subopercular ring has become greatly enlarged.

#### The Apical Apparatus of *Humaria hemisphaerica*

Asci containing immature ascospores are 160-240  $\mu\text{m}$  long and 12-16  $\mu\text{m}$  wide. They appear thick-walled except at the apex (Figs. 19, 20). In mature asci, which are long and cylindric (280-340 x 16-20  $\mu\text{m}$ ) the tips have become notably thicker (Fig. 28). An inner layer is detected in the opercular and subopercular region of the ascus after staining of

one-micron plastic sections with toluidine blue. Subapically, a faint bulge is detectable at the level of the first spore (Fig. 28). In asci that are about to rupture (Fig. 27), a hyaline ring delimits the operculum. The ascus appears stretched and thinner-walled at this stage.

The fine structure of ascal tips at early spore wall formation (Fig. 21) shows the ascal wall to be relatively thin (190-210 nm) in the immediate region of the tip. At the level of the first spore the wall increases to 520-550 nm in thickness. During ontogeny, the asci and paraphyses are embedded in a mucilaginous matrix (Figs. 21-26, 29-33). As the ascospores continue to mature (Fig. 22), the wall dimensions stay approximately the same. Within 3.1-3.5  $\mu\text{m}$  of the apex an annular protuberance is faintly detected on the inner surface of the ascal wall (Fig. 22). At spore maturity (Figs. 23, 25), this subapical ring becomes slightly more defined. The thickness of the apical and lateral ascal walls has not changed. A large vacuole exists throughout most of the ascus except at the tip which remains filled with cytoplasm (Fig. 23). Figure 24 represents a later time in development. The apical and subapical walls have increased in thickness to 310-350 nm and 700-750 nm, respectively, due to the addition of an inner layer (Fig. 26). The subapical ring, which is stained by silver methenamine, appears to be confluent with the inner layer (Fig. 26). At a subsequent stage, the inner layer, irregular in outline, thickens to 140-180 nm in the vicinity of

the operculum (Figs. 29, 30, 31). The subapical or subopercular ring is observed infrequently at this time (Fig. 26). Demarcation of the operculum is not apparent in any developmental stage of intact asci. At ascal dehiscence, the dislodged operculum is observed occasionally (Fig. 33), having a diameter of 5.8-6.2  $\mu\text{m}$  and a breadth of 380-420 nm. The suboperculum is 4.9-5.2  $\mu\text{m}$  long.

#### The Apical Apparatus of *Sphaerosporella brunnea*

The mature ascus is broadly cylindric (150-190  $\mu\text{m}$  x 16-20  $\mu\text{m}$ ). The rounded to blunt tip appears thinner-walled than the rest of the ascus (Fig. 35). The diameter of the ascospores (13-16  $\mu\text{m}$ ) is greater than that of the ascal tip (9-10  $\mu\text{m}$ ). Consequently, the first ascospore rests near the tip but is not closely appressed against the operculum. At spore release the operculum remains attached to one side of the ascus (Fig. 36). The ascostome appears to have a wider diameter than that of the operculum, which at this time is 5-6  $\mu\text{m}$ . The lateral ascal wall collapses and folds as well.

Ultrastructurally, the tip of a mature ascus consists of a flattened, thin wall, 420-470 nm thick, which broadens to 680-740 nm towards the ascal base (Figs. 37, 38). When examining potassium permanganate-fixed material (Fig. 37), the wall layers are sufficiently distinguished. The outer layer is composed of a rough, electron-dense external stratum, which broadens from 45-65 nm subapically to 130-150 nm at the apex, and an electron-transparent internal stratum



(Figs. 37, 39). In general, the outer layer increases in thickness from 280-320 nm throughout the opercular region of the ascus to 500-550 nm at the base of the suboperculum which is marked by the presence of a subopercular ring (Figs. 37, 39, 41). The subopercular ring is 450-480 nm long and consists of an electron-dense band, 45-55 nm thick, which has been deposited within the inner layer against the internal stratum of the outer layer. Like the outer layer, the inner layer thickens from 130-150 nm at the tip to 190-220 nm at the level of the subopercular ring (Fig. 37). Asci fixed in a glutaraldehyde-paraformaldehyde solution and poststained in lead citrate and uranyl acetate do not demonstrate wall layering (Fig. 38). Slight bulges (arrows) indicate the locality of the subopercular ring. Treating the thin sections with silver methenamine sharply defined the two wall layers (Figs. 40, 41). More importantly, the intense staining of the internal stratum of the outer layer in the operculum distinguishes this region from the suboperculum (Fig. 40). The suboperculum is 9.7-10.4  $\mu\text{m}$  long and tapers in thickness from 680-740 nm at the level of the subopercular ring to 390-420 nm near the operculum. The operculum is 4.8-5.2  $\mu\text{m}$  in diameter, which compares closely with the diameter of opercula seen in dehisced asci under the light microscope. In dehisced asci (Fig. 42), the lower extremity of the suboperculum is 380-400 nm thick. The wall immediately below the subopercular ring, however, has a thickness of 550-580 nm. It would appear that prior to

spore release the suboperculum becomes greatly stretched and remains so after spore release.

#### The Apical Apparatus of *Aleuria aurantia*

During early spore formation, the ascal wall appears thin at the tip and progressively becomes thicker toward the base (Fig. 43). At a later stage in development, the ascus, which is more inflated at the apex, is seen to be slightly heliotropic (Fig. 44). By maturity the ascus reaches a length of 200-240  $\mu\text{m}$  and a diameter of 12-16  $\mu\text{m}$ . No distinctive features are observed in the rounded ascal tip (Fig. 51). At ascal dehiscence, the operculum, 4-5  $\mu\text{m}$  in diameter, remains partially affixed to the lateral wall (Fig. 52). The diameter of the ascostome is roughly similar in size to that of the operculum.

Ultrastructural observations of asci approaching the end of ascospore development reveal thick lateral walls, 400-420 nm, which taper at the apices to a thickness of 140-170 nm (Fig. 45). At 4.0-4.4  $\mu\text{m}$  below the tip, the wall protrudes into the ascoplasm, forming a subopercular ring. A plasmalemmasome of considerable size is associated with the early development of this ring (Figs. 45, 46). When stained with silver methenamine at this stage of development, the ascal wall consists primarily of a thick outer layer (Fig. 47) and a thin (25-35 nm thick), strongly stained inner layer. The small plasmalemmasome in Fig. 47 is also stained intensely. The formation of the subopercular ring appears to be initiated asymmetrically (Fig. 48). At times

the subopercular ring becomes prominent, being 800-840 nm thick and 320-420 nm long (Figs. 49, 50). The ascal tip is filled with long, laminated endoplasmic reticulum (Figs. 45, 46, 49) which is associated with mitochondria, lipid bodies, ribosomes and small vesicles.

The apical wall of the mature ascus broadens to 280-300 nm (Fig. 54). Staining with silver methenamine demonstrates that the increase in thickness is due to the thickening of the inner layer, which is 140-160 nm throughout the opercular and much of the subopercular regions (Fig. 53). The outer layer is 100-130 nm thick apically and increases to 280-300 nm toward the base of the ascus. A subopercular ring was never detected at this stage in development (Fig. 53). At incipient ascal dehiscence (Fig. 55), the inner layer of the operculum is pulled away from the suboperculum. The opercular boundary is observed only at this time and in dehiscent asci (Fig. 56). The operculum has a diameter of 4.0-4.2  $\mu\text{m}$  and a uniform thickness of 260-290 nm. After spore release (Fig. 57), the suboperculum, which is 5.8-6.2  $\mu\text{m}$  long, thickens from 210-240 nm at the distal end to 360-440 nm at the level where the subopercular ring was previously observed. The inner layer broadens from 110-120 nm to 170-190 nm for a short distance in the lower extremity of the suboperculum.

#### The Apical Apparatus of *Anthracobia melaloma*

Mature asci are cylindric, 160-200  $\mu\text{m}$  x 10-14  $\mu\text{m}$  (Fig. 58). The ascal wall appears to be thicker throughout the

lateral face of the ascus and thinner at the tip (Fig. 59). Near the ascal tip a slight protuberance of the lateral wall is observed occasionally (Fig. 59). An apical funnel and continuous tract, which lead to the first spore, have been detected in asci that were placed in Congo red or aniline blue. One-micron sections stained in toluidine blue (Fig. 60, arrows) exhibited a subapical ring or band.

Electron microscopic observations of asci at early spore wall formation show that a localized band starts to form at 3.2-3.8  $\mu\text{m}$  from the tip (Fig. 61, arrows). Lomasomal activity is seen throughout the apical region of the ascal wall at this stage in spore development. As the spores continue to mature (Figs. 62, 63) the subopercular band becomes more conspicuous, consisting of an electron-dense area, 560-600 nm long and 50-60 nm thick. When treated with silver methenamine (Fig. 62) only the internal face of the subopercular band is stained. The wall at the immediate region of the tip is 140-170 nm thick. Subapically, the ascal wall reaches a thickness of 250-290 nm by a distance of 200-250 nm from the tip and stays at that thickness for the remainder of the ascus (Fig. 63). Near the end of ascosporogenesis, the inner layer is formed throughout the ascal wall (Fig. 64). Lomasomes are again observed, being most abundant in the vicinity of the apex. The subopercular band develops into a distinct, swollen ring. At spore maturity, formation of the inner layer is completed (Figs. 65, 66, 67, 68). The area of the operculum is delimited by its greater

thickness, 240-270 nm, from the distal end of the suboperculum. Most of the thickness of the operculum is due to the outer layer being 165-190 nm (Figs. 66, 68). The subopercular outer layer measures 65-100 nm at its upper extremity but increases to 210-220 nm by the level of the subopercular ring. In its entirety, the suboperculum, which is 6.8-7.2  $\mu\text{m}$  long, broadens from 190-210 nm next to the operculum to 350-380 nm just below the subopercular ring (Figs. 65, 67). The inner layer of the suboperculum is divided into an electron-transparent internal stratum and an electron-opaque external stratum (Fig. 67). The demarcation between the two strata is sharper than between the outer layer and the inner layer's external stratum. This study treats the external stratum as a part of the inner layer rather than refers to it as a separate layer for the following reasons: (1) The separation of the internal and external strata of the inner layer cannot be made developmentally. The formation of both strata is a continuous action. On the other hand, there is a period when wall development ceases to occur between outer layer and the external stratum of the inner layer. (2) The external and internal strata of the inner layer are combined below the operculum. Consequently, stratification of the inner layer is not observed in the operculum. When staining with silver methenamine the inner layer reacts positively within the operculum. The internal stratum of the inner layer also is richly stained throughout the suboperculum. The outer boundary of the inner layer's external

stratum is stained, delimiting the inner layer in this region of the ascal wall.

The Apical Apparatus of *Scutellinia scutellata*

Young asci at the eight-nucleated to early spore stages are cylindric to subcylindric, 100-150  $\mu\text{m}$  x 8-12  $\mu\text{m}$ . The ascal wall, which stains strongly in Congo red, is distinctly thin at the tip (Fig. 69). Mature asci (Fig. 70) reach a length of 200-260  $\mu\text{m}$  and a diameter of 14-18  $\mu\text{m}$ . The ascal wall appears to be uniformly thick in the apical and sub-apical regions (Fig. 77). No distinctive features are observed in the mature tip. At ascospore discharge, the operculum is partially attached to the ascus (Fig. 78). The lateral walls appear flaccid and typically collapse.

Ultrastructurally, the apex of a four-nucleated ascus is thin-walled, 130-160 nm, and stains strongly in silver methenamine (Figs. 73, 75). Numerous, small to large vesicles are scattered throughout the upper half of the ascus (Fig. 73). Large lomasomes are frequently observed in both the apical and lateral regions of the ascal wall (Fig. 75). The lateral wall is comparably thick, 240-255 nm, and stains weakly in silver methenamine in its outer stratum. A thin mucilaginous coat, 65-75 nm, covers only the tip of the ascus (Fig. 75). In the eight-nucleated ascus (Fig. 74), the mucilaginous coat increases to a thickness of 210-240 nm at the tip and extends to the base of the ascus. An exaggerated lomasomal-like band forms below the tip at this



stage in development. The innermost portion of the wall in this zone reacts positively in silver methenamine (Fig. 74).

At early spore wall formation, the tip, which remains thin-walled, 140-180 nm, is filled with small vesicles and endoplasmic reticulum (Figs. 71, 76). At 5.6-5.9  $\mu\text{m}$  below the tip a subopercular ring protrudes inwardly from the lateral wall (Fig. 71). In Fig. 72 the annular protuberance has an irregular, lomasomal appearance. After treatment with silver methenamine, the innermost portion of the ascal wall is stained as well as its outermost portion (Fig. 76, arrows). By spore maturity, the internally stained region of the ascal wall in Fig. 76 has developed into an intensely stained middle layer (Figs. 79, 80, 81), which decreases from 110-130 nm in thickness at the upper extremity of the ascus to 45-65 nm toward the base. In the vicinity of the previously existing subapical protuberance, the middle layer is weakly stained for a length of 520-540 nm (Fig. 79). The ascal tip has broadened to 370-390 nm. The increase is basically due to the production of an inner layer which is 130-150 nm thick throughout the opercular region and tapers to 30-40 nm at the level of the subopercular ring (Figs. 79, 80). Sections that are stained with uranyl acetate and lead citrate exhibit a thick mucilaginous coat, 540-650 nm (Fig. 82). The inner and middle wall layers are weakly differentiated. Delimitation between the mucilaginous coat and the outer layer is extremely difficult. At the initiation of ascal dehiscence, the middle and outer

layers are pulled apart along a zone of dehiscence between the operculum and suboperculum (Figs. 81, 83). The operculum at this stage has a diameter of 6.8-7.0  $\mu\text{m}$ . The lower extremity of the subopercular wall has thickened to 470-490 nm.

#### The Apical Apparatus of *Ascozonus woolhopensis*

Before ascospore delimitation, asci reach a length of 80-160  $\mu\text{m}$  and diameter of 14-18  $\mu\text{m}$ . They are broadly clavate and possess a conical tip (Fig. 84). Below the tip, a swollen ring protrudes from the thinner side of the ascal wall. By maturity, the ascus increases an additional 20-40  $\mu\text{m}$  in length and 4-6  $\mu\text{m}$  in diameter. The ring and apical wall appear more pronounced in thickness (Fig. 85). When placed in Congo red, the subapical ring and outer layer of the ascal wall are strongly stained (Fig. 85). The immediate region of the tip remains hyaline. Ascal dehiscence occurs by two or three transverse fissures (Fig. 94) that originate at the tip.

Ultrastructurally, young asci during early ascosporogenesis (Fig. 86) are conspicuously thick-walled. The apical wall, being faintly conical, consists of two broad layers and is bordered by a prominent subapical ring. The outer layer is thinner narrowing from 550-610 nm at the apex to 320-280 nm at the ring. The inner layer similarly decreases from 900-950 nm at the apex to 350-400 nm just above the ring. At the ring the two layers are indistinct. The ring is 1250-1300 nm thick. The lateral wall below the ring is 1300-1350 nm thick, being mostly comprised of a thickened

inner layer, 1000-1050 nm (Fig. 86). In Fig. 87, a stage comparable to Fig. 84, the apical region of the ascus is distinctly conical. The outer layer and most of the subapical ring are intensely stained by silver methenamine (Figs. 87, 91). The inner layer appears to be stratified for a distance of 2.4-2.8  $\mu$ m above the ring where demarcation of the sublayers is weakened toward the tip. At the ring the inner layer remains unstained, forming a thin, transparent band, 25-35 nm, which broadens to 750-830 nm below the ring. The inner layer expands from a thickness of 680-720 nm above the ring to 1790-1850 nm at the apex. A small pore is formed at the tip during this stage of development (Fig. 88). The outer layer and a middle stratum broaden from 320-350 nm above the ring to 450-480 nm below the tip. From there the outer layer tapers to 130-190 nm at the tip. The mature apices basically exhibit no differences in form from that shown in Fig. 87. The thicknesses of the lateral and subapical walls are reduced by 360-400 nm and 250-280 nm, respectively, while the ring stays at 1300-1350 nm. The attenuation of the lateral and subapical regions indicates that the ascus wall is becoming stretched at this time. The middle stratum and the inner and outer layers are observed more clearly in peripheral sections (Fig. 89). Visual distinction of the wall layering at the subapical ring is extremely difficult to make in median sections. Peripheral sections of the ring show that the middle stratum comprises most of that region (Figs. 90, 93), thus reinforcing the

layering distinguished by the silver methenamine stain in Figs. 87 and 91.

In dehisced asci, an apical disc (Fig. 92), consisting mostly of the outer layer, is observed. The electron-transparent inner layer appears to have shrunk or dissolved at this stage. The disc has a diameter of 2.4-2.6  $\mu\text{m}$  and a thickness of 550-600 nm.

#### The Apical Apparatus of *Geopyxis majalis*

Mature asci are narrow and cylindric, 260-300  $\mu\text{m}$  x 10-14  $\mu\text{m}$ . The ascal wall appears to be uniformly thick at the apex and lateral sides (Fig. 95). Asci with immature spores have thinner apical walls (Fig. 96). At dehiscence the spores are released through a wide ascostome (Fig. 96) with the operculum being completely detached.

Electron microscopic examinations of mature asci demonstrate a bilayered apical wall (Fig. 97). The layers are strongly accentuated when treated with silver methenamine (Fig. 99). The inner and outer layers of the opercular wall are identically 170-190 nm thick. At the periphery of the operculum the outer layer forms a bulge increasing in thickness to 210-240 nm for a length of 270-300 nm while the inner layer decreases to 85-95 nm in this region (Fig. 99, arrows). The inner layer briefly thickens to 125-160 nm below the bulge before tapering to 25-40 nm at 3.2-4.0  $\mu\text{m}$  below the apex. Conversely, the outer layer briefly thins to 125-160 nm below the bulge before it broadens to 320-340 nm toward the base of the ascus. Younger asci do not

display a distinctly stained inner layer (Fig. 98). The distal region of the ascus is filled with cytoplasm. The walls of immature and mature asci are covered with a mucilaginous coat (Figs. 98, 99). At incipient ascus dehiscence, the subopercular and opercular inner layers are pulled apart (Fig. 101), thus delimiting the operculum. During spore release the upper extremity of the suboperculum is tightly pressed against the sides of the spore as the spore passes through the ascostome (Fig. 100).

#### The Apical Apparatus of *Sowerbyella imperialis*

The mature ascus is long and cylindric, 180-200  $\mu\text{m}$  x 10-13  $\mu\text{m}$ . The wall appears thin throughout the ascus, staining lightly in Congo red (Fig. 102). The operculum, having a diameter of 5.5-6.5  $\mu\text{m}$ , is observed with the aid of phase-light microscopy.

Ultrastructurally, the mature ascus is extremely thin-walled and flaccid (Figs. 104, 105, 108), being 220-260 nm thick both apically and laterally. The inner layer increases from 20-30 nm laterally to 90-110 nm at the vicinity of the tip while the outer layer decreases from 170-200 to 120-140 nm. A mucilaginous coat (Figs. 104, 107) covers the entire ascus. When stained in silver methenamine the inner layer reacts more intensely (Figs. 106-109). An operculum is not readily distinguished except when the inner layer becomes thinner at an area below the tip (Fig. 108). The operculum has a diameter of 6.2-6.7  $\mu\text{m}$ . At spore release,

the suboperculum, which is 4.6-5.1  $\mu\text{m}$  long, has increased an additional 40-60 nm (Fig. 109).

### Discussion

Light microscopic examination of the ascal tips of species in the Otidea-Aleuria complex revealed general uniformity in form and development. Except in Ascozonus woolhopensis, the apical wall remained thin throughout the later stages of ascosporogenesis. Apical apparatuses of all members but Anthracobia melaloma and A. woolhopensis lacked distinct features by which they could be characterized. The indented ring reported in Chapter I was not detected in any species. Asci of A. melaloma and A. woolhopensis possessed subapical, swollen rings. In A. melaloma the ring was observed in mature ascal walls after close inspection. By comparison, the ring in A. woolhopensis developed much earlier and became quite pronounced. Prior to ascal dehiscence the operculum was seldom seen in the mature asci of the ten representatives, having been observed only in Humaria hemisphaerica and Sowerbyella imperialis. Van Brummelen (1974) demonstrated an apical disc in the ascus of A. woolhopensis with the aid of the stain Congo red and thus substantiated earlier findings of Vuillemin (1887). Kimbrough (1972), however, pointed out that the nipped tips in asci of Ascozonus cunicularius (Boud.) Marchal were unstained in Congo red. He did not observe a disc or lid in asci of that species. Similarly, the apical apparatus of



A. woolhopensis in the present study did not exhibit an apical disc in fresh material that had been stained with Congo red. Observations of A. cunicularius (Kimbrough, 1972) and A. woolhopensis in this study were made from material that was collected in North America. Staining properties of the small disc may vary according to species and isolates of Ascozonus.

Chadefaud (1942) described delicate cytoplasmic components, which varied in size and complexity, for the apical apparatuses of eight members placed within the Otidea-Aleuria complex (Figs. 110A, B, C, D). Two of these species, Aleuria aurantia and Humaria hemisphaerica, were currently studied. Components including the apical punctuation, tract and funnel were not seen in stained and unstained fresh material of either species. Of the remaining species in the present examination, Anthracobia melaloma has a funnel and tract in the apical region of its ascus. These features were vague and infrequently observed. Apical spherules were seen in most species from time to time but without regularity.

Opercular pads reported by LeGal (1953) in species of Phaedropezia and Trichophaea were found in the apical apparatuses of Otidea leporina. Optical view of the mature ascal tip in O. leporina seemed to show an expanded opercular wall. Fine structure of the apex, however, demonstrated the opercular wall to be thinner than the subtending lateral wall and have a convex shape. Thus, the structurally weak ascal tips of O. leporina, seen with the light microscope,

frequently became invaginated during mounting or staining procedures. This phenomenon was misleading in the present study and may have misled LeGal in her analysis of Phaedropezia and Trichophaea.

The distinct light microscopic feature of the different ascus tips in the present investigation was the thickness of the ascus walls. Subapical walls of asci in O. leporina, Sphaerospora brunnea, Jafnea fuscarpa and Humaria hemisphaerica appeared considerably thicker than those seen in A. melaloma, Scutellinia scutellata, Geopyxis majalis and Sowerbyella imperialis. In multispored asci (more than eight per ascus) of A. woolhopensis the walls were extremely thick and displayed two layers. A bilayered ascus wall was also observed in H. hemisphaerica and J. fuscarpa.

Ultrastructural observations of the ten species presently studied reinforced the light microscopic findings. Thickness of the lateral ascus walls was sharply divided between those that were thin, 350-450 nm, as in Aleuria aurantia, Anthracoia melaloma, Geopyxis majalis, Scutellinia scutellata and Sowerbyella imperialis and those that were thick, 700-1100 nm, as in Ascozonus woolhopensis, Humaria hemisphaerica, Jafnea fuscarpa and Sphaerospora brunnea. Otidea leporina was the only representative that did not fall into either division. Gross morphology of the apical apparatuses was similar for all by A. melaloma, O. leporina and S. brunnea. The outer layer decreased in thickness toward the tip while the inner layer decreased toward the

base. In A. melaloma, O. leporina and S. Brunnea the inner layer thickened toward the base or stayed approximately at the same thickness throughout the ascal wall.

With the exception of A. woolhopensis, the development of the apical apparatuses essentially followed the three-step sequence that was outlined in Chapter I. In two species, J. fusicarpa and H. hemisphaerica, the inner layer was formed after spore ontogeny. Their pattern of development resembled that seen in Iodophanus granulipolaris Kimbr. Formation of the apical apparatus in A. woolhopensis differed significantly in several respects. The inner layer of the lateral ascal wall was formed before ascospore delimitation. By early ascosporogenesis the development of the apical apparatus was complete. At this stage a pore had formed in the inner layer at the ascal tip. Van Brummelen (1974) noted the appearance of the pore at a later time in development and suggested that it was formed from a process of localized disintegration. The pore may have been, however, the result of wall stretching as the ascus expanded in length and width throughout ascosporogenesis. A similar phenomenon was observed in the thick-walled apex of Cookeina sulcipes (Berk.) Kuntze (Samuelson, 1975).

Schrantz's (1970) interpretation of the development of the ascal wall in Tarzetta cupularis differed significantly from that shown in the present study. He stated that in young asci the exoascus consisted of a thin band of electron-dense, fibrillar material and by spore formation, this layer

had increased in thickness mostly at the tip. He concluded that most of the wall consisted of a thick endoascus which tapered in thickness toward the tip. Apparently, Schrantz did not examine fully ripened asci as an examination of his photographs will confirm. His descriptions of exo- and endo-ascas walls of T. cupularis corresponded to the mucilaginous coat and outer layer, respectively, of a number of species including S. scutellata, S. imperialis, and A. woolhopensis.

Cytoplasmic components of the operculate apical apparatus described by Chadeffaud (1942) (Fig. 110A) were not detected ultrastructurally in any of the representatives currently examined. Chadeffaud's discovery of the funnel and tract were most likely artefacts of fixation and staining. He frequently applied either Melzer's reagent or a chromium trioxide-osmium tetroxide solution when examining the apical apparatus. These solutions may have caused the collapse of the ascus plasmalemma and tonoplast beneath the apical region of the ascus, thus creating a funnel and tract. Tips of most representatives in the present study were filled with cytoplasm throughout ascosporeogenesis. The apical cytoplasm may account for the presence of apical punctuations observed by Chadeffaud above the funnel (Fig. 110A).

An interesting wall component that was pointed out by Chadeffaud (1942) in several species was the occurrence of a subapical "bourrelet," i.e. pad. In the general scheme of the operculate apical apparatus (Fig. 110A) the subapical pad adjoined the operculum. However, his diagrams of dehiscent

asci in Aleuria aurantia and Scutellinia hirta (Fig. 110B, C) depicted subapical pads that were further removed from the tip. Subapical pads were not currently seen with the light microscope in A. aurantia or S. scutellata. Ultrastructurally, a subopercular, asymmetrically formed, swollen ring was discovered in A. aurantia during late spore development. Although the ring had disappeared by maturity, the subopercular wall was thickest in that vicinity. In S. scutellata a subopercular ring was also observed with the aid of the electron microscope. The subapical pads described by Chadeffaud may have represented subopercular rings. He noted that the pads were often asymmetrical as in A. aurantia and depicted them in only mature and dehiscent asci. S. hirta and A. aurantia were both studied by Chadeffaud in a chromo-osmic solution. Wall layering in that region of the ascus may have been abnormally affected by this solution, thus permitting observation of the pads.

Among the ten members of the Otidea-Aleuria complex presently studied, seven species displayed subopercular rings. First appearance of the ring occurred at different developmental stages for different species. In J. fusicarpa an annular protuberance was detected after the inner layer was formed. By comparison, the first signs of a subopercular ring in A. melaloma were observed prior to the formation of the inner layer. In general, development of the annular swelling occurred during the last stages of ascosporeogenesis and marked the initiation of the formation of the inner

layer. However, in A. woolhopensis the ring began to develop shortly after meiosis in the young, truncate ascus. Similar observations of A. woolhopensis were made by van Brummelen (1974). Still, both investigations demonstrated that the ring was derived from the local expansion of the inner layer.

Van Brummelen's interpretation of the wall composition in A. woolhopensis at the ring and throughout the apical region of the ascus differed from the present study in three respects. (1) He recognized the presence of a middle layer in the subapical wall which disappeared at the level of the ring. This layer is currently described as part of the middle stratum which extends to the base of the ascus. Median sections of younger asci stained with silver methenamine and peripheral sections of mature asci established this finding. (2) Van Brummelen demonstrated an electron-dense internal layer at the level of the ring. Since he was not able to follow it above or below the ring, he referred to the area as the internal ring layer. Staining with silver methenamine revealed that the internal ring layer was a part of the inner layer. (3) The present study also reports the presence of a distinct middle stratum within the inner and outer layers. The ring was shown to consist mostly of the middle stratum (Fig. 111H) which appears to be chemically similar to the outer wall layer. Similar findings were observed with the light microscope using Congo red. Van Brummelen did not make these distinctions.



The smallest subopercular rings occurred in the thick-walled species, J. fusicarpa, H. hemisphaerica and S. brunnea. In contrast, taxa that formed conspicuous rings during the development of their apical apparatuses, i.e., A. aurantia and A. melaloma, were thin-walled. A. woolhopen-sis was the sole exception, having both thick walls and an enormous ring. Multispored asci, in general, have been shown to be thick-walled (Kimbrough, 1966a,b, 1969; Kimbrough and Korf, 1967). In Chapter I wall dimensions of the 32-spored representative Thecotheus pelletieri (Crouan) Boud. were roughly 3 times that of the 8-spored members in the iodine-positive group. In Chapter III the apical apparatus of the multispored species Coprotus winteri (Marchal) Kimbr. was shown to be essentially an enlarged replica of the eight-spored species Coprotus lacteus (Ck. and Phill.) Kimbr. Similarly, wall dimensions of A. woolhopen-sis were approximately two and one-half to three times that of A. melaloma and A. aurantia. Therefore, when taking the exaggerated condition of the multispored ascus into consideration, the apical apparatus of A. woolhopen-sis is remarkably similar in form to that of A. melaloma.

Kimbrough and Benny (1977) have recently described in another multispored representative, Lasiobolus monascus Kimbr., the presence of a large subopercular ring during the development of its apical apparatus. As in Aleuria aurantia, the ring, which became most prominent during ascosporangogenesis, was not apparent at the end of spore development.

Stretching of the ascal wall prior to spore release may have been responsible for its disappearance.

Within the Otidea-Aleuria complex the function of the subopercular ring appeared to be associated primarily with structural support of the apical region of the ascus during spore release. At incipient ascal dehiscence, the lateral wall became markedly stretched, narrowing its thickness by 10-20%. In the species A. melaloma, A. aurantia, S. brunnea and S. scutellata, where the subapical walls decreased significantly in thickness toward the tip, the ring most likely provided additional strength and maintained the shape of the ascal tip. During dehiscence, the structural role that the ring played was most evident in A. woolhopensis, keeping the lateral wall intact as the spores shot through the fissured opening. After dehiscence, in certain members, J. fusicarpa, O. leporina and H. hemisphaerica, the lateral wall appeared to be elastic, having returned to its original thickness. In S. brunnea elasticity of the subopercular wall was not observed. The wall above the subopercular ring remained stretched. Although a distinct subopercular ring was not found in mature asci of O. leporina, the sharp increase in wall thickness at the lower extremity of the suboperculum may have provided strength comparable to that of the localized rings in S. brunnea and A. melaloma.

Apical apparatuses of Geopyxis majalis and Sowerbyella imperialis demonstrated general similarities with the other taxa. Although revived material was used, wall layering of

the ascal tip was readily distinguished in both species. The presence of a ring at the periphery of the operculum in G. majalis was unique among the representatives of the Otidea-Aleuria complex examined. The thinness of the ascal wall in S. imperialis was equally atypical. Subopercular rings were not observed in either representative. Since most of the subopercular rings currently studied have been shown to be inconspicuous, developmental examinations of fresh specimens are necessary to verify the presence or absence of this component within the ascal wall.

The present examination has demonstrated that within the members studied no two species shared identical apical apparatuses. Ascal tips consisted primarily of thin-walled and thick-walled forms. When comparing the thick-walled ascus of J. fusicarpa (Fig. 111E) to the thin-walled ascus of A. melaloma (Fig. 111G), the differences were distinct. However, the species H. hemisphaerica, S. brunnea (Fig. 111F) and A. aurantia exhibited intergrades between the two. Defined types of apical apparatuses within the Otidea-Aleuria complex could not be made on the basis of wall thickness or any other feature of the ascal tip including the subopercular ring.

Except in Ascozonus woolhopensis, Lasiobolus monascus and spp. of Thelebolus subapical swollen rings have not been observed outside of the Otidea-Aleuria complex. Variations of the size of this unusual wall component correlated most closely with wall thickness and, to a lesser degree, with

the spore number. Presence of subopercular rings in A. woolhopensis and L. monascus indicated possible taxonomic affinities with members of the Otidea-Aleuria complex. Both species have been placed in the Thelebolaceae, based primarily on their multispored condition (see Chapter V). In a world monograph of Lasiobolus, Bezerra and Kimbrough (1975) stated that developmental, morphological, and microchemical properties of Lasiobolus were more related to Cheilymenia, Coprobia and Scutellinia than to Thelebolus. They suggested that Lasiobolus belonged in the tribe Scutellinieae sensu Korf (1973). The present study was supportive of their findings. Cytological and developmental studies on Ascozonus are needed to understand more clearly its taxonomic position within the operculate Discomycetes. Of the operculate apical apparatuses that have been ultrastructurally examined in present and previous examinations, A. woolhopensis most clearly resembled Anthracobia melaloma in both structure of the ascal wall and general form.

Apical apparatuses of the Otidea-Aleuria ascus were found to be morphologically diverse. The diameter and thickness of the operculum, the length and thickness of the suboperculum, the stratification of the wall, the size and shape of the subopercular ring and the ontogeny of the different components varied from taxon to taxon. Still, several characters of the apical apparatuses were generally common for the representatives presently studied. A distinct dehiscent region observed in the iodine-positive ascus

(see Chapter I), the eugymnohymenial ascus (see Chapter III) and the suboperculate ascus (Samuelson, 1975; van Brummelen, 1975) was infrequently found in the Otidea-Aleuria complex. The protruding apical wall in O. leporina and the thickened apical wall in Anthracobia melaloma were the only examples of morphologically delimited opercula. Differential staining of the operculum with silver methenamine, which was described in Iodophanus granulipolaris Kimbr. (see Chapter I), Pyronema domesticum (Sow. ex Fr.) Sacc. and several representatives of the Sarcoscyphineae (Samuelson, 1975), occurred only in S. brunnea. The differences of the staining intensities of the opercular wall most likely signify changes in the wall chemistry at that area. Other operculate species may have opercula that differ chemically from the rest of the ascal wall but do not exhibit the differences when staining with silver methenamine or lead citrate and uranyl acetate. Further examination of ascal walls is needed to find various treatments that could reveal this feature.

A fibrillar zone of dehiscence was not detected in the ascal tips of any of the species in the present study. On the other hand, the subopercular ring was a common feature of the wall of the Otidea-Aleuria ascus. All of the members that were investigated developmentally demonstrated to some extent the presence of this component. The similarity of the apical apparatuses and their subopercular rings in S. brunnea and A. melaloma quite possibly suggests closer taxonomic relatedness between these taxa than shown in

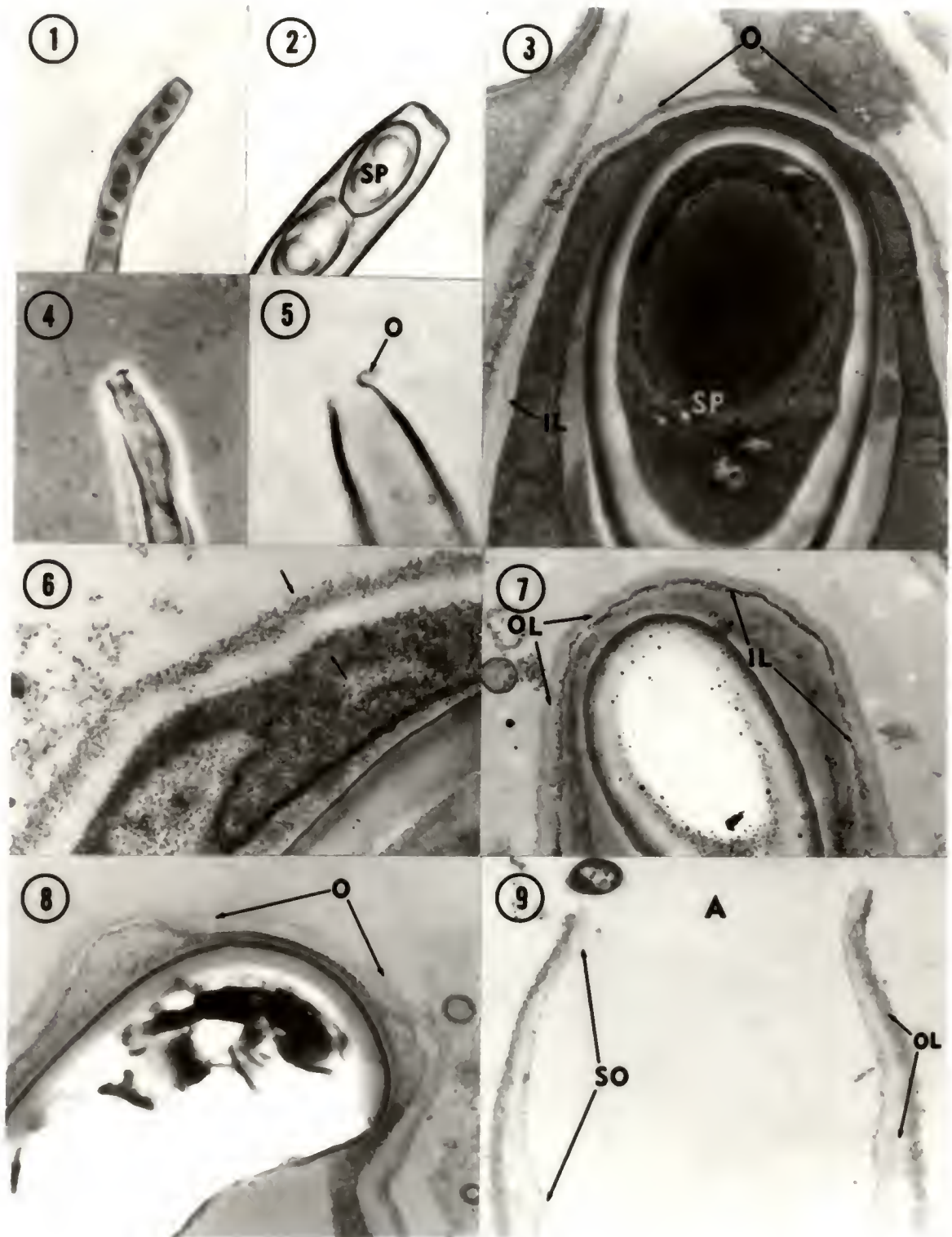
Table 1. Additional studies of the apical apparatuses in the Otidea-Aleuria complex are needed to learn of the variability and prevalence of the subopercular ring. The present study has shown that the apical apparatus may be useful as a systematic tool within this largest of the operculate groups.



## Chapter II

### Figures 1-9. Otidea leporina

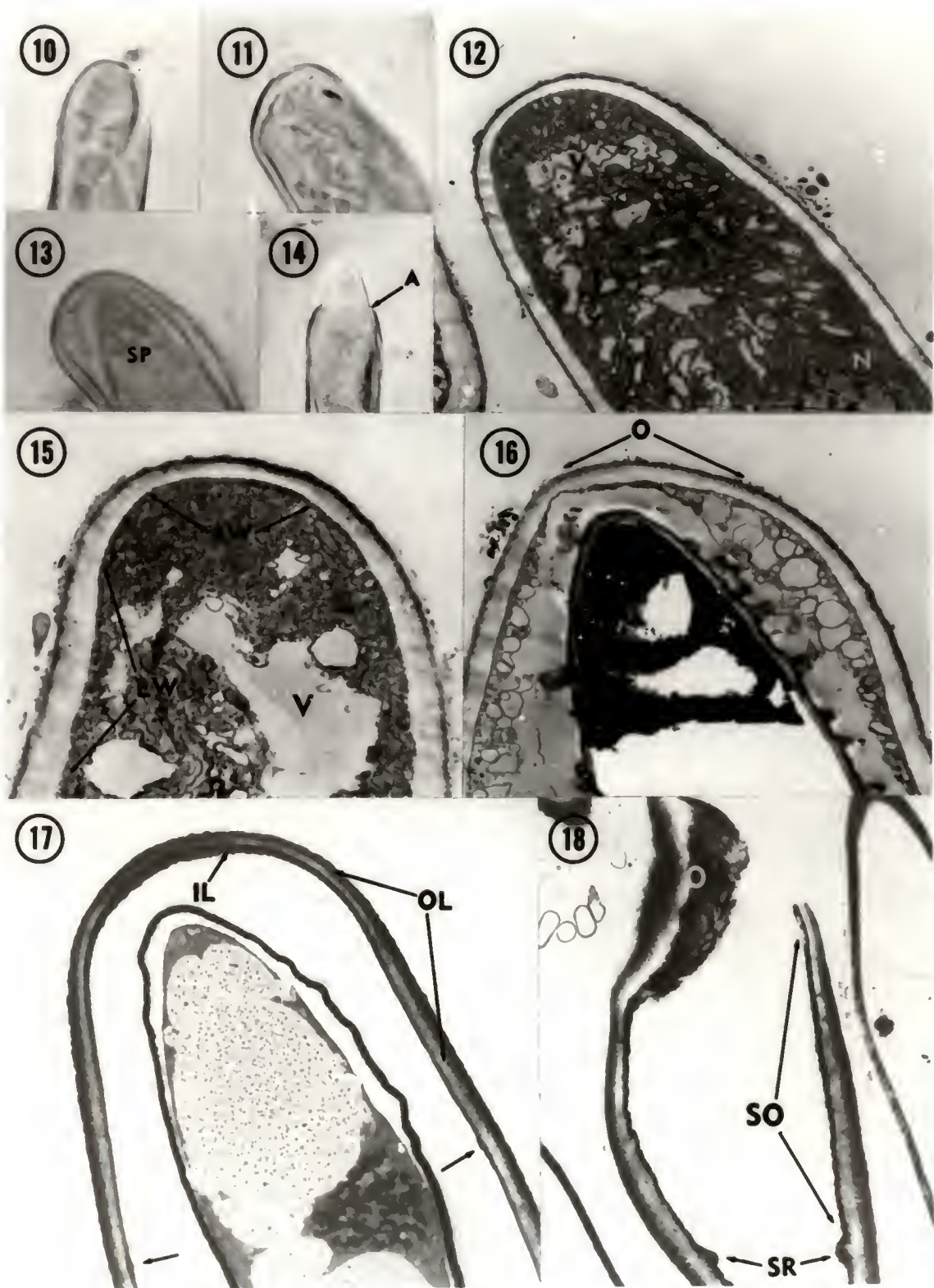
- Figure 1. Subapical region of mature ascus is distinctly heliotropic. X400.
- Figure 2. Tip of mature ascus. Spore (SP). Stained with Congo red. X1,500.
- Figure 3. Raised portion at tip of mature ascus, delimiting operculum (O). Inner layer (IL). Spore (SP). X14,000.
- Figure 4. Shrunken ascus after spore release. Phase contrast. X400.
- Figure 5. Dehiscent ascus with operculum (O) laterally attached. Stained with Congo red. X1,500.
- Figure 6. Distal portion of suboperculum. Arrows point to region where opercular dehiscence will occur. X37,000.
- Figure 7. Mature apical apparatus showing wall layering. Stained with silver methenamine. Inner layer (IL). Outer layer (OL). X10,000.
- Figure 8. Ascus tip at time of spore release. Operculum (O). X14,000.
- Figure 9. Dehiscent ascus showing shrunken suboperculum (SO). Ascospore (A). Outer layer (OL). X15,000.



## Chapter II

### Figures 10-18. Jafnea fusicarpa

- Figure 10. Ascal tip during early spore formation. Stained with Congo red. X1,000.
- Figure 11. Apical and subapical walls at later time in development. Stained with Congo red. X1,000.
- Figure 12. Apical region of four-nucleated ascus with scattered small vesicles (V). Nucleus (N). X4,000.
- Figure 13. Mature ascal tip showing slight heliotropism. Spore (SP). X1,000.
- Figure 14. Ascospore is compressed within ascostome (A) during its ejection. X500.
- Figure 15. Ascal tip during early spore wall development shows increased thickness at lateral walls. Apical wall (AW). Vesicle (V). X6,700.
- Figure 16. Broadened ascal tip at end of spore development. Operculum (O). X6,200.
- Figure 17. Mature apical apparatus with small subapical, swollen ring (arrows). Inner layer (IL). Outer ring (OL). Stained with silver methenamine. X5,000.
- Figure 18. Dehisced ascus with attached operculum (O). Suboperculum (SO). Subopercular ring (SR). Stained with silver methenamine. X5,400.

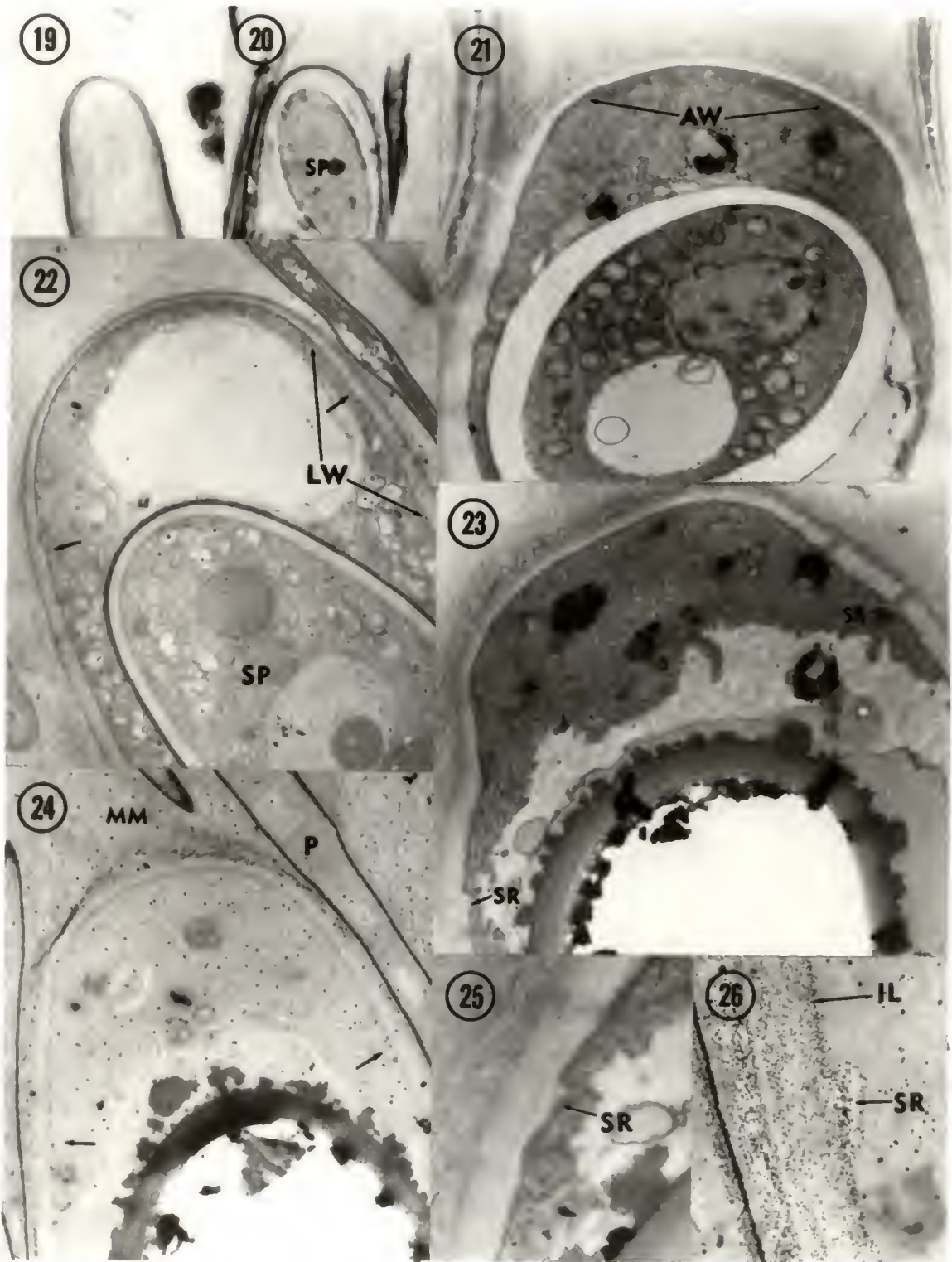


## Chapter II

### Figures 19-26. Humaria hemisphaerica

- Figure 19. Young ascus tip during spore development. Stained with Congo red. X1,000.
- Figure 20. Ascus tip with developing spores (SP). One-micron section stained with crystal violet. X1,000.
- Figure 21. Thin apical wall (AW) of ascus at early spore wall formation. X7,800.
- Figure 22. Ascus tip at later stage in spore (SP) development. Arrows point to annular protuberance at lateral wall (LW). X6,000.
- Figure 23. Apical region of ascus remains filled at end of spore development. Subapical ring (SR). X7,500.
- Figure 24. Later developmental stage of apical apparatus. Arrows point to subapical ring. Mucilaginous matrix (MM). Stained with silver methenamine. X5,000.
- Figure 25. Portion of the lateral wall showing small subapical ring (SR). X17,000.
- Figure 26. Region of subapical ring (SR) stained with silver methenamine. Inner layer (IL). X21,000.



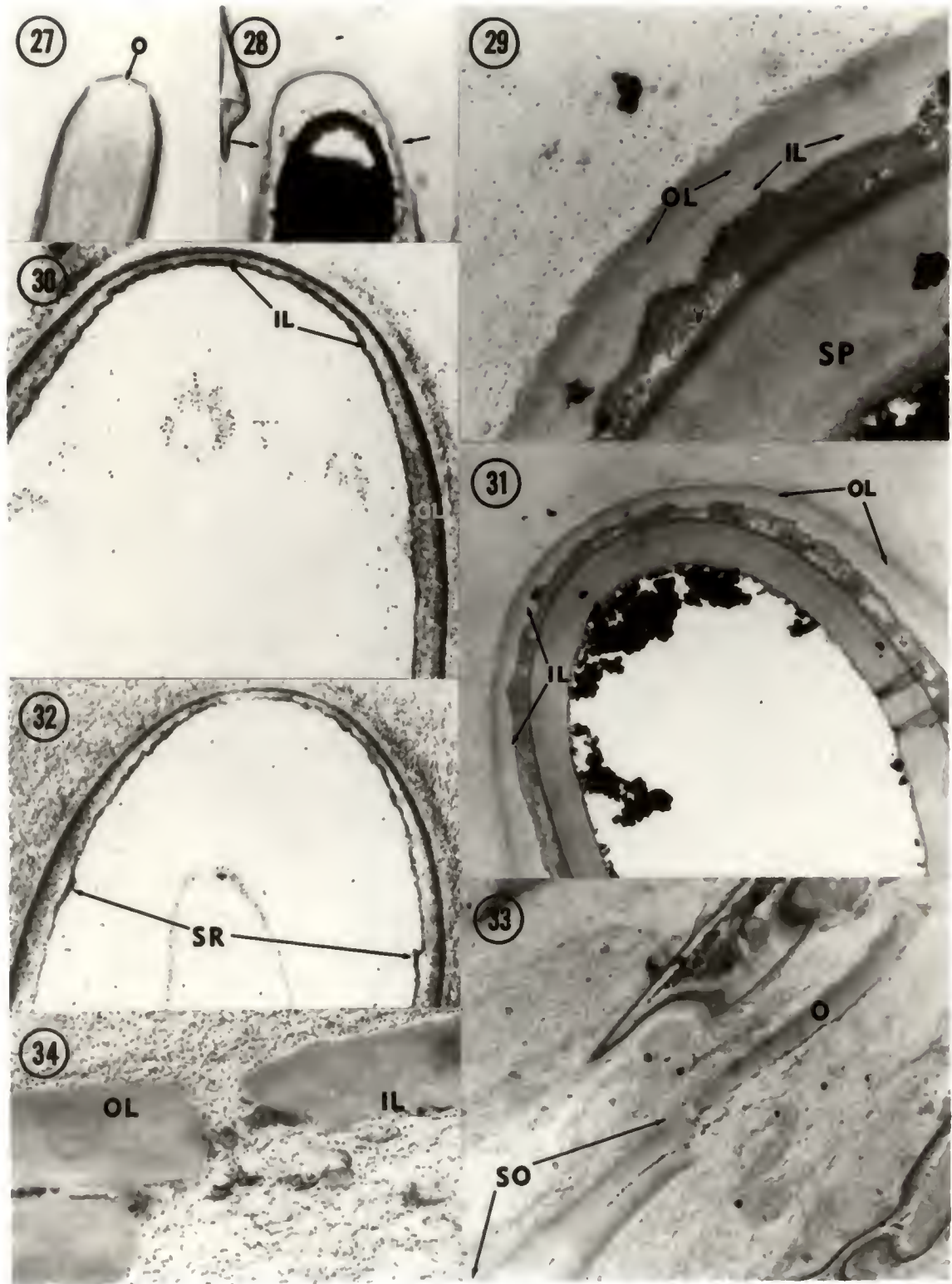




## Chapter II

### Figures 27-34. Humaria hemisphaerica

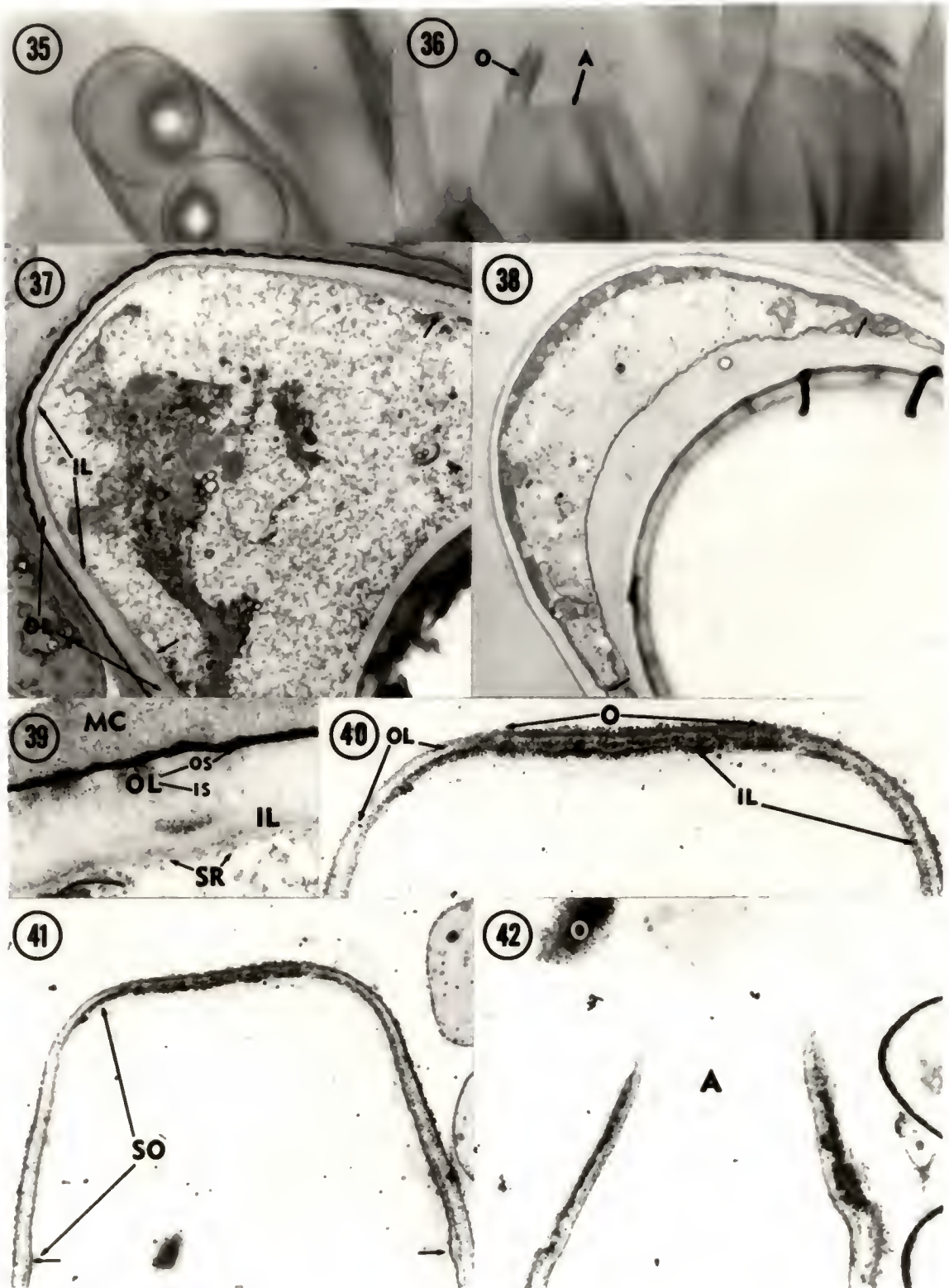
- Figure 27. Mature apical apparatus at incipient spore release. Operculum (O). Stained with Congo red. X1,000.
- Figure 28. One-micron section of mature ascus tip shows faint subapical ring (arrows). Stained with toluidine blue. X1,000.
- Figure 29. Opercular region of mature ascus tip. Inner layer (IL). Outer layer (OL). Spore (SP). X32,000.
- Figure 30. Mature ascus tip stained with silver methenamine. Inner layer (IL). Outer layer (OL). X9,000.
- Figure 31. Ascospore pressed against tip of mature ascus. Inner layer (IL). Outer layer (OL). X8,200.
- Figure 32. Occasional protruding subapical ring (SR) in mature ascus. Stained with silver methenamine. X7,700.
- Figure 33. Dehiscent ascus with dislodged operculum (O). Suboperculum (SO). X8,200.
- Figure 34. Border of suboperculum and operculum showing expanded inner layer (IL). Outer layer (OL). X30,000.



## Chapter II

### Figures 35-42. Sphaerosporella brunnea

- Figure 35. Mature ascus with thin apical wall. Stained with Congo red. X1,000.
- Figure 36. Opercula (O) are partially attached during spore release. Stained with Congo red. Ascostome (A). X1,000.
- Figure 37. Tip of mature ascus that has been fixed in permanganate. Arrows point to subapical band at level of first spore. Inner layer (IL). Outer layer (OL). X9,000.
- Figure 38. Tip of mature ascus fixed in glutaraldehyde-paraformaldehyde solution. Arrows point to subopercular ring. X6,900.
- Figure 39. Region of subopercular ring (SR) showing an outer (OS) and inner stratum (IS) of the outer layer (OL) and an inner layer (IL). Mucilaginous coat (MC). X26,000.
- Figure 40. Operculum (O) is demarcated by intense staining of the outer layer (OL). Inner layer (IL). Stained with silver methenamine. X10,000.
- Figure 41. Ascal tip showing suboperculum (SO), operculum and suboperculum ring (arrows). Stained with silver methenamine. X5,000.
- Figure 42. Dehisced ascus with stretched subopercular wall. Stained with silver methenamine. Ascostome (A). Operculum (O). X8,300.

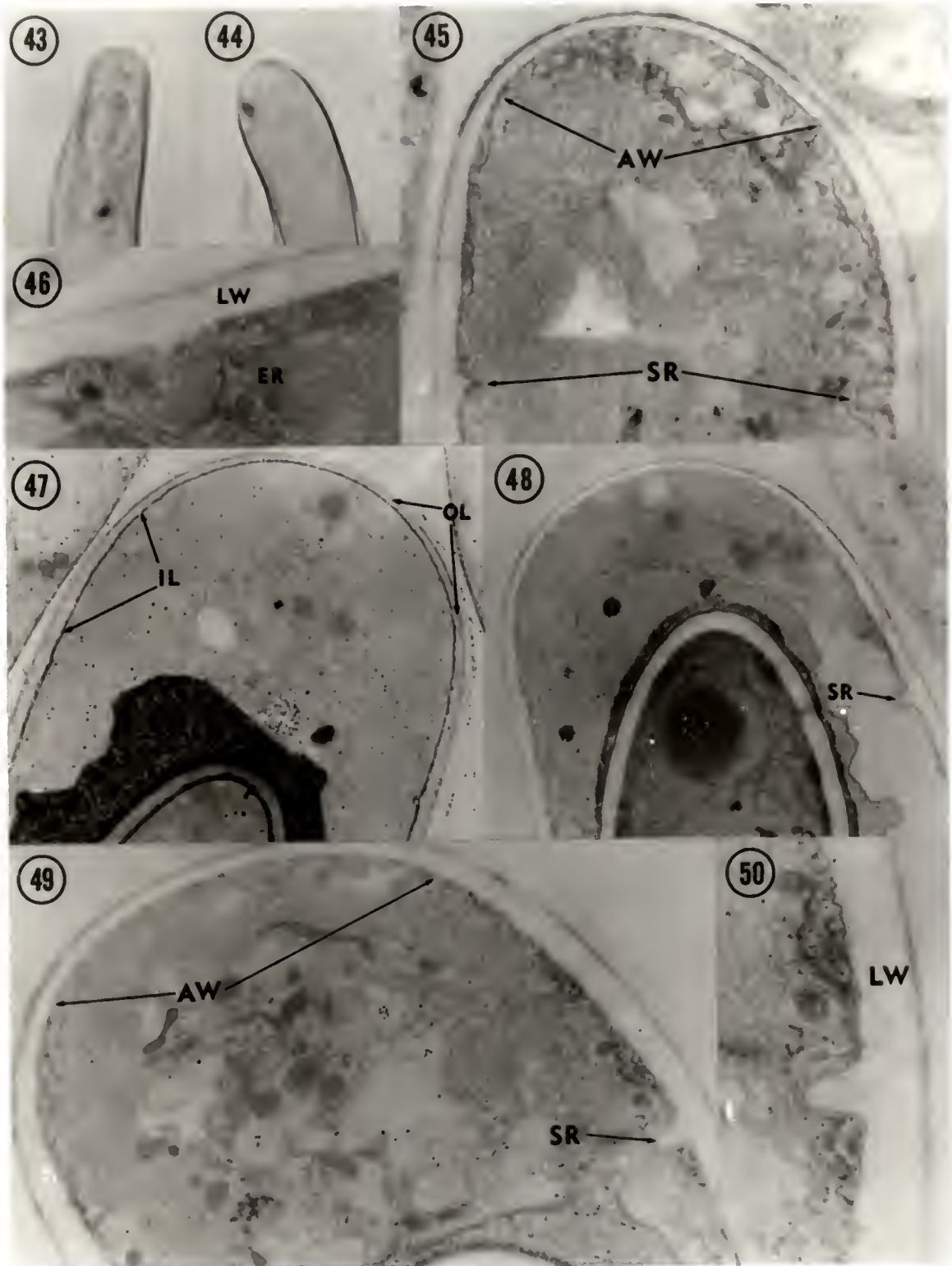




## Chapter II

### Figures 43-50. Aleuria aurantia

- Figure 43. Ascus during early spore formation with thin-walled apex. Stained with Congo red. X1,000.
- Figure 44. At later stage in spore development, ascus becomes slightly heliotropic near the tip. Stained with Congo red. X1,000.
- Figure 45. Ascus tip during spore wall development shows early formation of subopercular ring (SR). Apical wall (AW). X10,500.
- Figure 46. Plasmalemmasome is associated with development of the ring. Lateral wall (LW). Endoplasmic reticulum (ER). X18,500.
- Figure 47. Presence of thin inner layer (IL) during spore wall development. Outer layer (OL). Stained with silver methenamine. X8,200.
- Figure 48. Asymmetrical formation of subopercular ring (SR). X7,500.
- Figure 49. Ascus tip filled with long, laminated endoplasmic reticulum. Apical wall (AW). Subopercular ring (SR). X13,000.
- Figure 50. Prominent subopercular ring shown in Fig. 49. Lateral wall (LW). X25,000

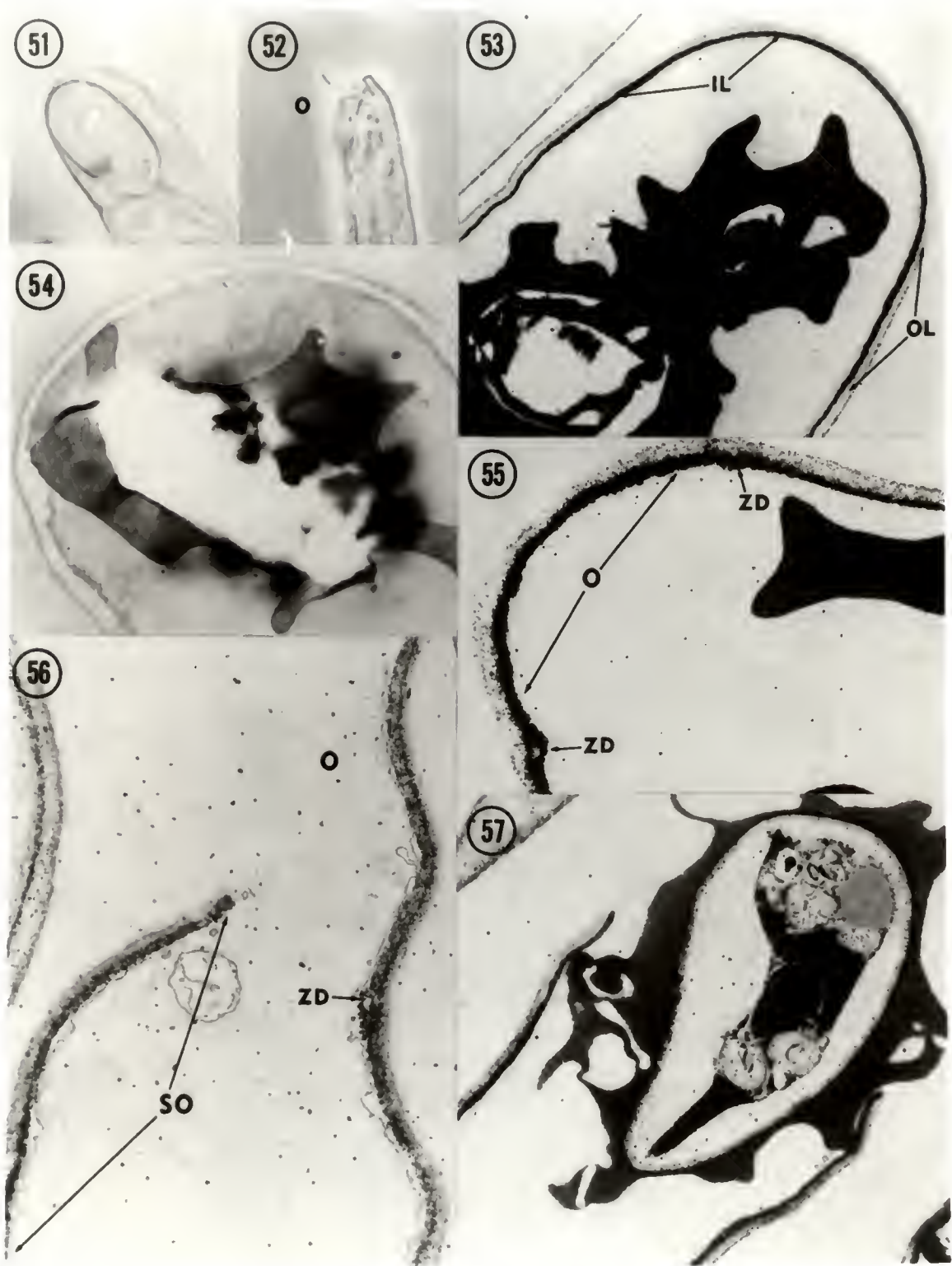




## Chapter II

### Figures 51-57. Aleuria aurantia

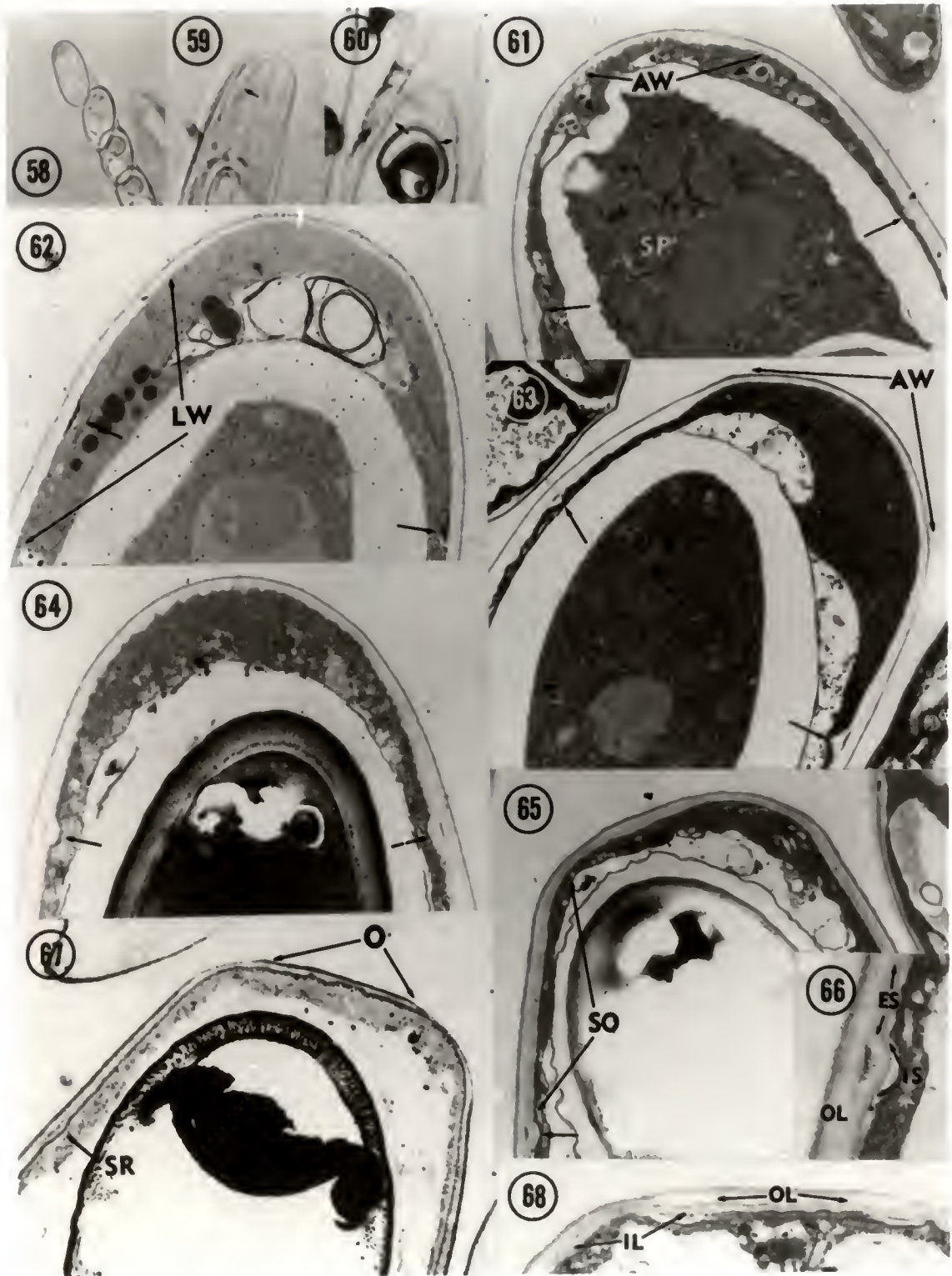
- Figure 51. Rounded tip of mature ascus lacks distinctive features. Stained with Congo red. X1,000.
- Figure 52. Attached operculum (O) at spore release. Phase contrast. X400.
- Figure 53. Mature tip with thickened inner layer (IL). Outer layer (OL). Stained with silver methenamine. X7,600.
- Figure 54. Wall layering in tip of mature ascus is poorly distinguished when stained with lead citrate and uranyl acetate. X10,000.
- Figure 55. Zone of dehiscence (ZD) and delimitation of the operculum (O) is seen at onset of spore release. Stained with silver methenamine. X12,500.
- Figure 56. Dehisced ascus with partially attached operculum (O). Suboperculum (SO). Zone of dehiscence (ZD). Stained with silver methenamine. X15,000.
- Figure 57. Spore is held tightly by subopercular wall surrounding the ascostome during spore discharge. Stained with silver methenamine. X8,000.



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### Figures 58-68. Anthracobia melaloma

- Figure 58. Mature ascus stained with aniline blue. X400.
- Figure 59. Protuberance (arrow) of lateral wall below ascus tip. Stained with aniline blue. X1,000.
- Figure 60. Subapical ring (arrows) in mature ascus. One-micron section stained with toluidine blue. X1,000.
- Figure 61. Early formation of localized band (arrows) at lateral wall of ascus during spore development. Apical wall (AW). Spore (SP). X9,600.
- Figure 62. Subapical band (arrows) is stained intensely by silver methenamine. Lateral wall (LW). X9,000.
- Figure 63. Thickened lateral wall during progressive development of spores. Apical wall (AW). Arrows point to subapical band. X8,600.
- Figure 64. Toward end of spore development subopercular band becomes swollen ring (arrows). X8,600.
- Figure 65. Mature apical apparatus shows delimitation of suboperculum (SO). Arrows point to subopercular ring. X8,600.
- Figure 66. Region of subopercular ring. Outer layer (OL). External stratum (ES). Internal Stratum (IS). X22,000.
- Figure 67. Mature apical apparatus stained with silver methenamine. Operculum (O). Subopercular ring (SR). X8,600.
- Figure 68. Area of operculum showing outer layer (OL) and inner layer (IL). X15,500.

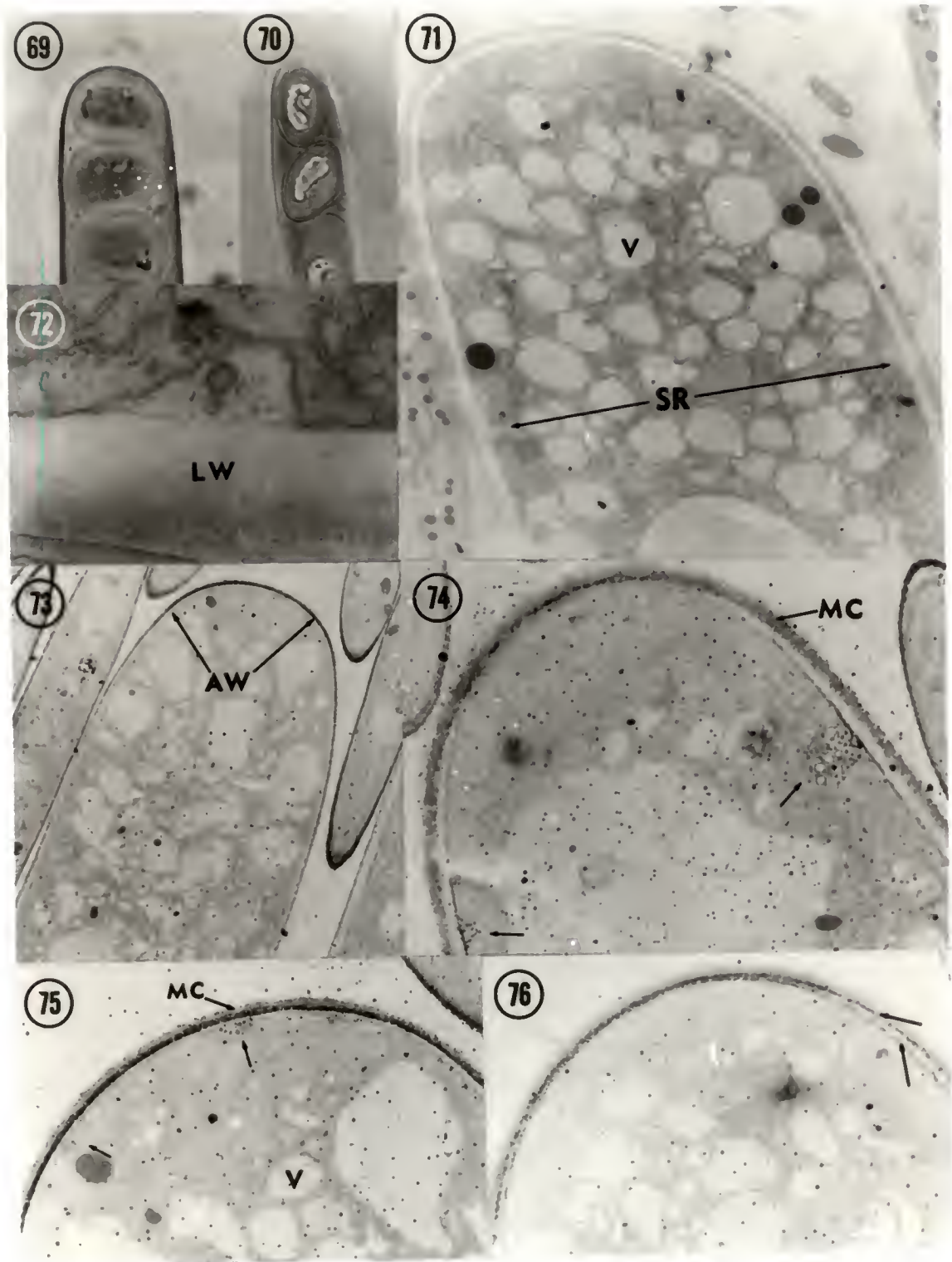


## Chapter II

### Figures 69-76. Scutellinia scutellata

- Figure 69. Young ascus during early spore wall formation. X1,000.
- Figure 70. Mature ascus stained with aniline blue. X300.
- Figure 71. Ascal tip at early spore wall formation shows numerous, small vesicles (V) and subapical ring (SR). X8,000.
- Figure 72. Area of subapical ring. Lateral wall (LW). X50,000.
- Figure 73. Four-nucleated ascus with strongly stained apical wall (AW). Stained with silver methenamine. X3,600.
- Figure 74. Apex of eight-nucleated ascus shows thickened mucilaginous coat (MC). Arrows point to lomasomal-like band. Stained with silver methenamine. X8,500
- Figure 75. Apex of four-nucleated ascus shown in Fig. 73. Arrows point lomasomes. Mucilaginous coat (MC). Vesicle (V). Stained with silver methenamine. X11,500.
- Figure 76. Apical wall of ascus during spore wall formation. Arrows point to stained regions of the wall. Stained with silver methenamine. X10,000.



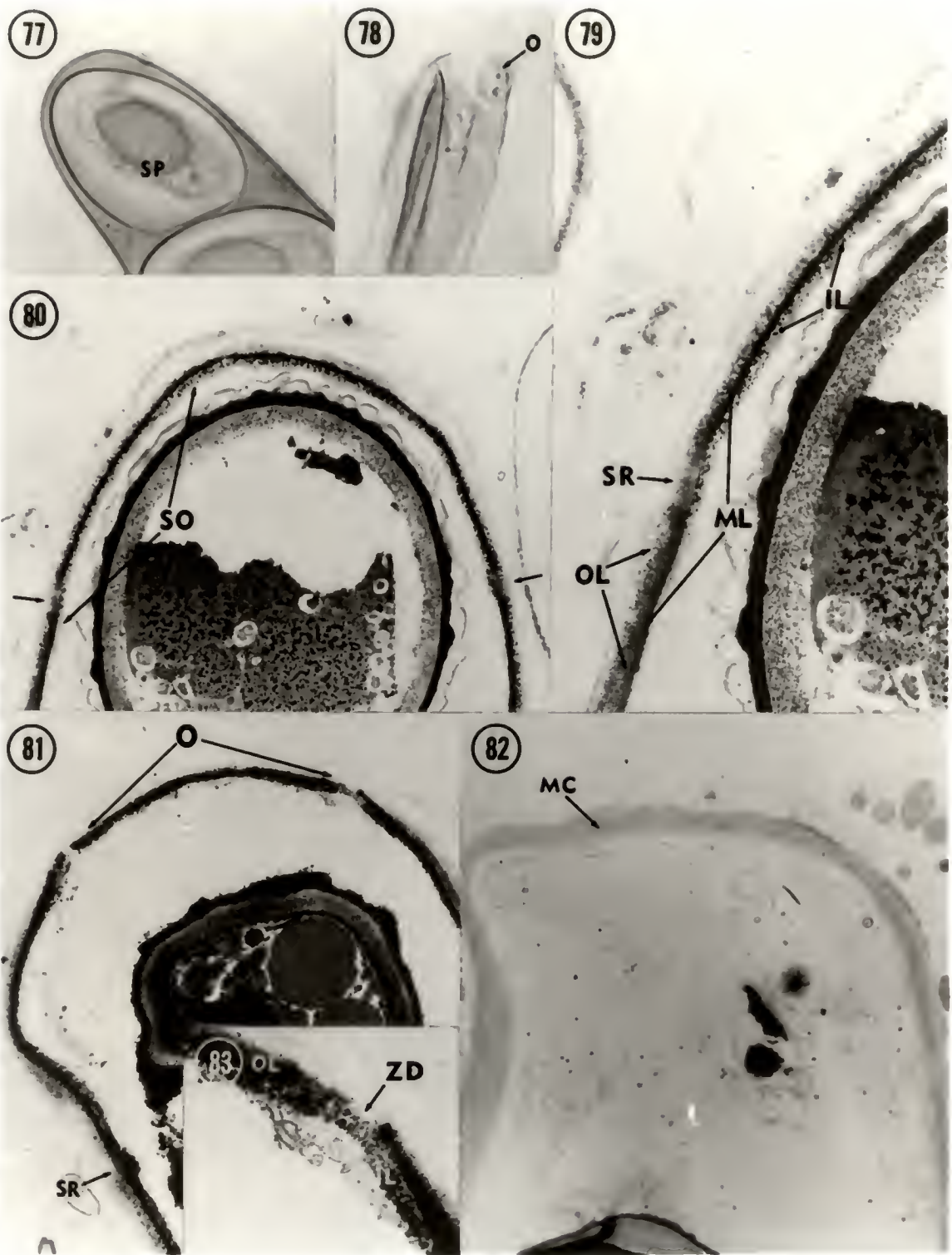




## Chapter II

### Figures 77-83. Scutellinia scutellata

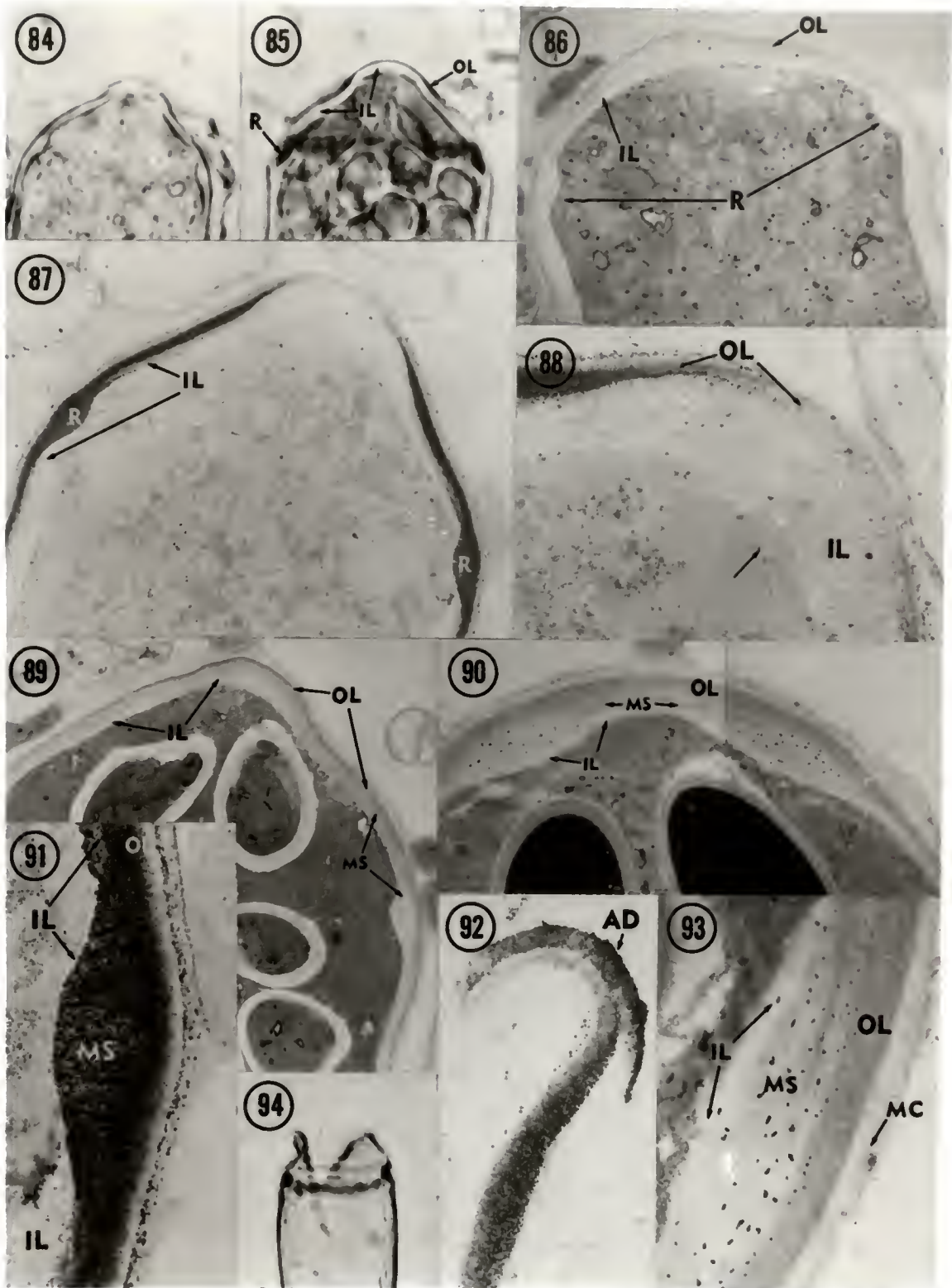
- Figure 77. Thin-walled tip of mature ascus. Stained with Congo red. Spore (SP). X1,000.
- Figure 78. Ascus at spore release with partially fastened operculum (O). X500.
- Figure 79. Subopercular region of mature ascus shows different layers and subopercular ring (SR). Inner layer (IL). Middle layer (ML). Outer layer (OL). Stained with silver methenamine. X14,000.
- Figure 80. Apical apparatus of mature ascus. Arrows point to subopercular ring. Suboperculum (SO). Stained with silver methenamine. X7,100.
- Figure 81. Ascus tip at incipient spore release. Operculum (O). Subopercular ring (SR). Stained silver methenamine. X7,300.
- Figure 82. Tip of mature ascus showing thick mucilaginous coat (MC). X8,900.
- Figure 83. Close-up of zone of dehiscence (ZD) in Fig. 83. Outer layer (OL). Inner layer (IL). Stained with silver methenamine. X21,000.



## Chapter II

### Figures 84-94. Ascozonus woolhopensis

- Figure 84. Tip of ascus before development of ascospores. Stained with aniline blue. X1,000.
- Figure 85. Mature tip stained with Congo red shows distinct subapical ring (R). Inner layer (IL). Outer layer (OL). X1,000.
- Figure 86. Forming apex of young ascus during early ascosporogenesis. Ring (R). Inner layer (IL). Outer layer (OL). X3,400.
- Figure 87. Ascal tip at later stage in development stained with silver methenamine. Inner layer (IL). Ring (R). X3,500.
- Figure 88. Small pore (arrow) at apex. Inner layer (IL). Outer layer (OL). Stained with silver methenamine. X11,000.
- Figure 89. Peripheral section of mature tip shows inner layer (IL), outer layer (OL) and middle stratum (MS). X3,500.
- Figure 90. Peripheral section of ring shows outer layer (OL), middle stratum (MS) and inner layer (IL). X6,000.
- Figure 91. Area of ring stained with silver methenamine. Inner layer (IL). Middle stratum (MS). Outer layer (OL). X15,000.
- Figure 92. Tip of dehisced ascus with apical disc (AD). Stained with silver methenamine. X11,000.
- Figure 93. Portion of ring seen in Fig. 90 shows different layers and mucilaginous coat (MC). Inner layer (IL). Outer layer (OL). Middle stratum (MS). X14,500.
- Figure 94. Ascus after spore release. Stained with Congo red. X400.

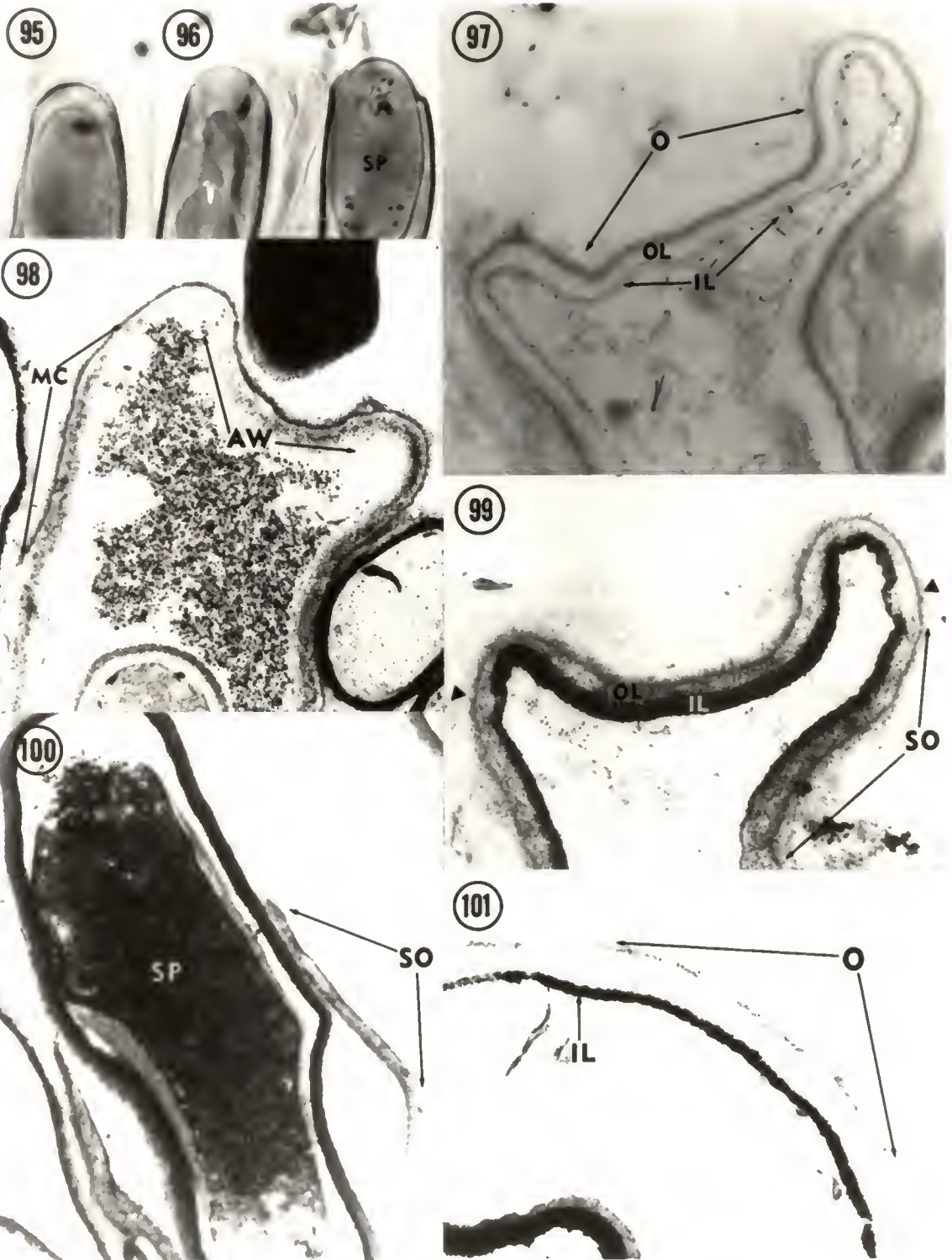


## Chapter II

### Figures 95-101. Geopyxis majalis

- Figure 95. Tip of mature ascus. Stained with Congo red. X1,000.
- Figure 96. Younger ascus with thin apical wall, and ascus at spore (SP) release. Stained with Congo red. X1,000.
- Figure 97. Apical wall of mature ascus shows inner layer (IL) and outer layer (OL). Operculum (O). X16,500.
- Figure 98. Ascus tip with slightly less developed ascospores. Apical wall (AW). Mucilaginous coat (MC). Stained with silver methenamine. X9,600.
- Figure 99. Tip of mature ascus stained with silver methenamine shows inner layer (IL) and outer layer (OL). Arrows point to apical ring. X15,500.
- Figure 100. Upper region of suboperculum (SO) pressing against spore (SP). Stained with silver methenamine. X8,500.
- Figure 101. Ascus tip at incipient spore release. Operculum (O). Inner layer (IL). Stained with silver methenamine. X8,500.

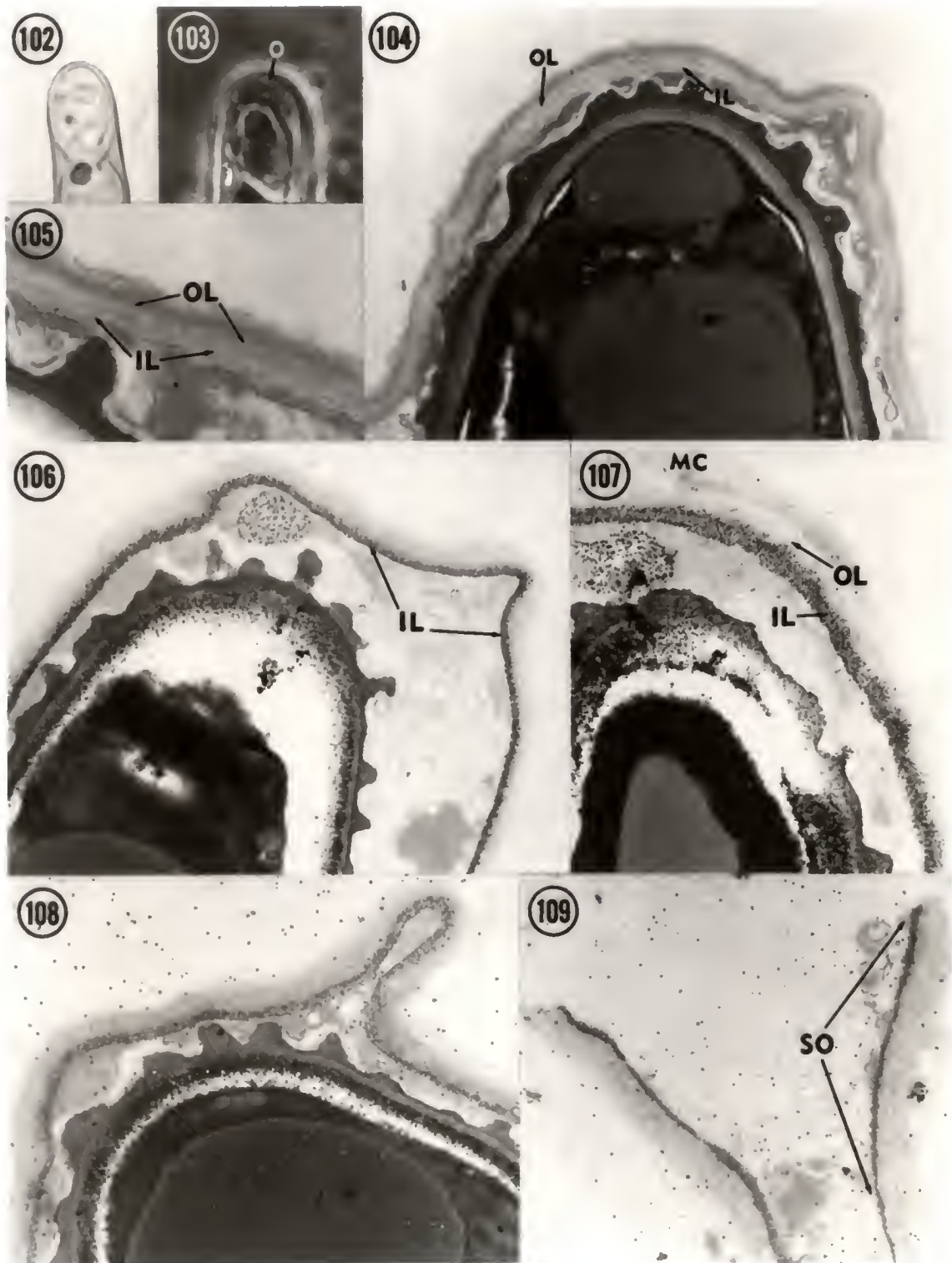




## Chapter II

### Figures 102-109. Sowerbyella imperialis

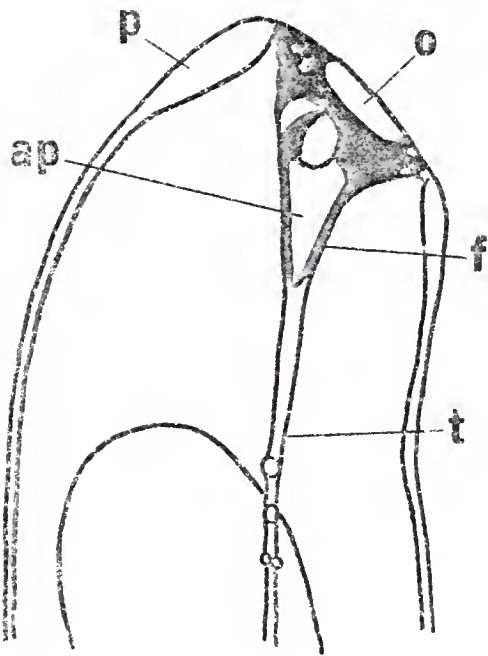
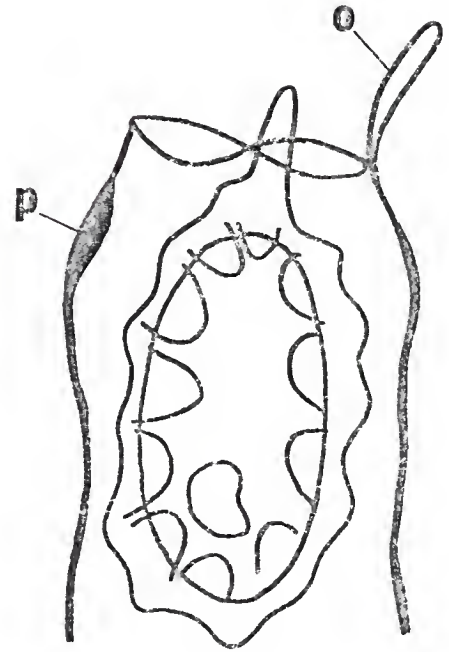
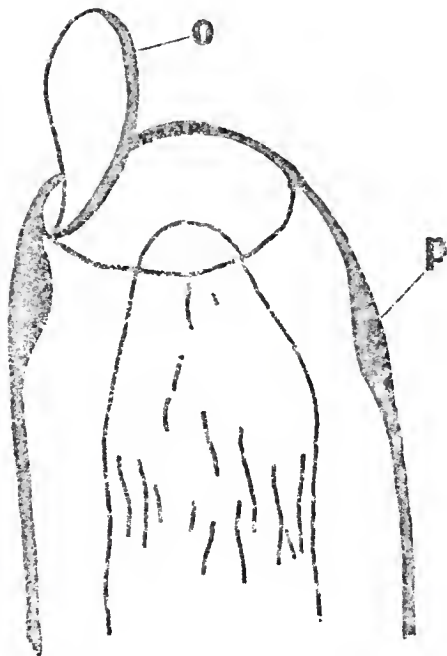
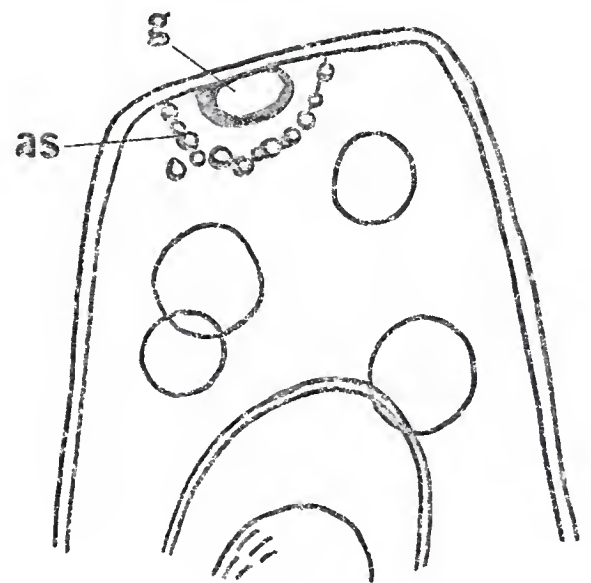
- Figure 102. Apical portion of mature ascus. Stained with Congo red. X1,000.
- Figure 103. Phase-contrast of mature tip. Operculum (O). X1,000.
- Figure 104. Thin-walled tip of mature ascus. Inner layer (IL). Outer layer (OL). X15,000.
- Figure 105. Portion of subapical wall showing inner layer (IL) and outer layer (OL). X31,000
- Figure 106. Mature ascus stained with silver methenamine. Inner layer (IL). X15,000.
- Figure 107. Subopercular region of mature ascus shows thickened inner layer (IL) and tapered outer layer (OL). Mucilaginous coat (MC). Stained with silver methenamine. X24,000.
- Figure 108. Ascal tip stained with silver methenamine. Arrows point to narrowed area of inner layer at apex. X17,000.
- Figure 109. Ascus after spore ejection. Suboperculum (SO). Stained with silver methenamine. X10,500.



## Chapter II

Figure 110. Apical apparatuses redrawn from Chadeffaud (1942).

- A. Operculate apical apparatus with complement of components. Apical punctuation (ap). Funnel (f). Pad (p). Operculum (o). Tract (t).
- B. Apical apparatus of Aleuria aurantia at spore release. Operculum (o). Pad (p).
- C. Apical apparatus of Scutellinia hirta at spore release. Operculum (o). Pad (p).
- D. Rudimentary apical apparatus of Octospora leucomata. Globule (g). Apical spherules (as).

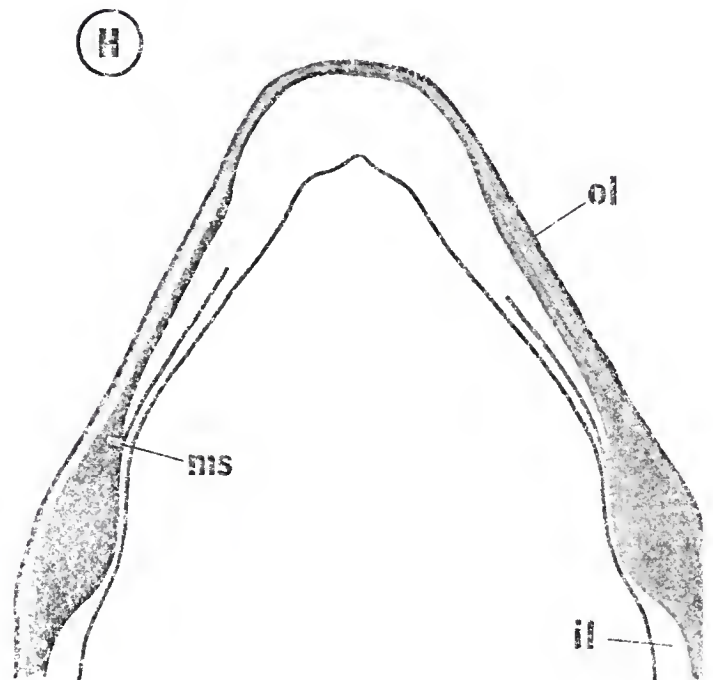
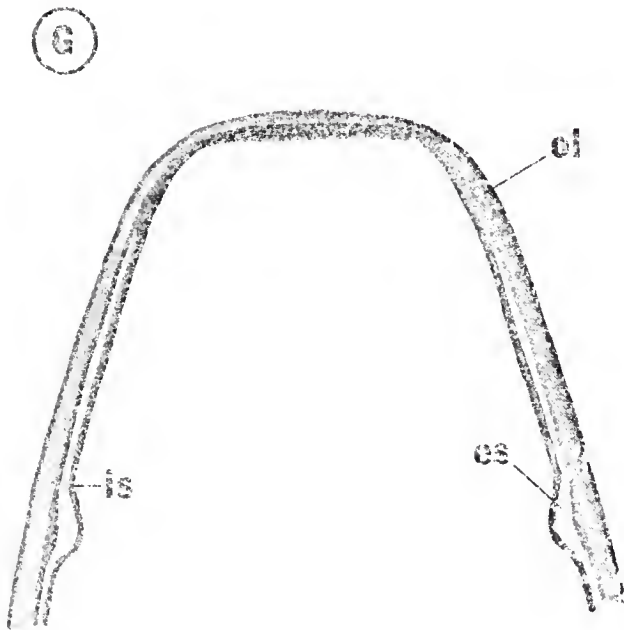
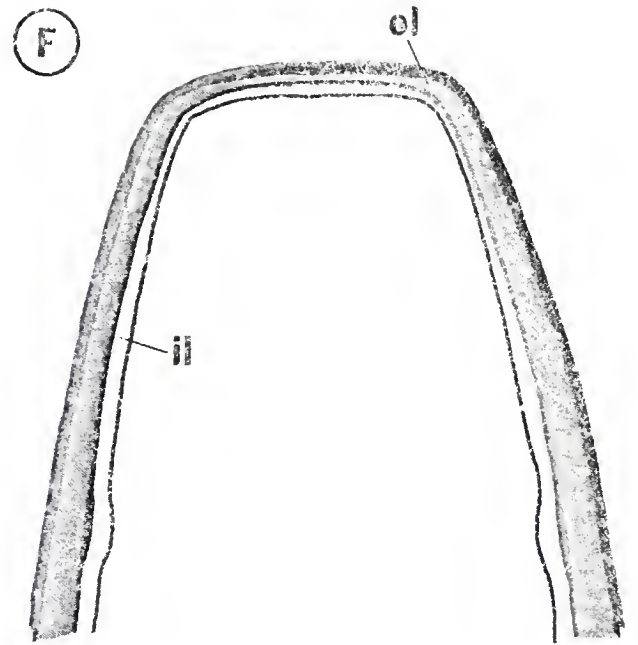
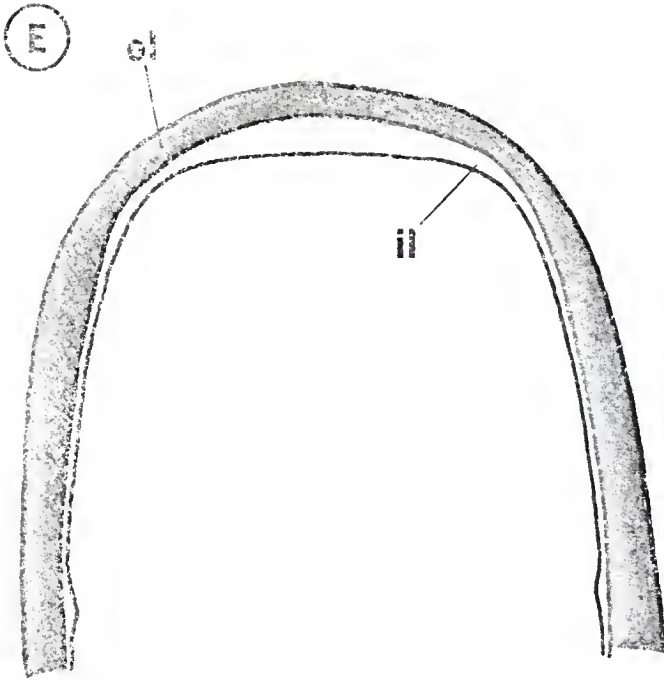
**A****B****C****D**



## Chapter II

Figure 111. Illustrations made from electron microscopic observations of apical apparatuses of the Otidea-Aleuria complex.

- E. Wall layering of mature ascal tip in Jafnea fusicarpa. Inner layer (il). Outer layer (ol).
- F. Wall layering of mature ascal tip in Sphaerosporella brunnea. Inner layer (il). Outer layer (ol).
- G. Wall layering of mature ascal tip in Anthacobia melaloma. Internal stratum (is) of inner layer. External stratum (ES) of inner layer. Outer layer (ol).
- H. Wall layering of mature ascal tip in Ascozonus woolhopensis. Inner layer (il). Middle stratum (ms). Outer layer (ol).



CHAPTER III  
MORPHOLOGY, DEVELOPMENT AND CYTOCHEMISTRY OF THE APICAL  
APPARATUSES OF EUGYMNHOHYMENIAL REPRESENTATIVES

Introduction

Within the operculate Discomycetes workers have traditionally distinguished two extreme types of apothecial ontogeny. Enclosed development of the hymenium has been referred to as "angiocarpic" (Corner, 1929; Snell and Dick, 1957). Development with the hymenium exposed has been referred to as "gymnocarpic" (Corner, 1930; Snell and Dick, 1957). For apothecia which first begin with an enclosed hymenium that later becomes exposed certain workers have used the term "hemiangiocarpic" (Singer, 1951; Jackson, 1949). Other workers (Corner, 1929; Reijnders, 1948) have included this latter type of development within the "angiocarpic" category. Van Brummelen (1967) established a set of terms clearly defining both stages and types of apothecial development. Ascoma that had an enclosed hymenium at least during its early formation was called cleistohymenial. Van Brummelen pointed out that most representatives within the operculate Discomycetes have this type of development. Exposure of the hymenium from the onset of ascocarp development until ascal maturation was called gymnohymenial. Few operculate members have been

shown to have this type of development. He subdivided this type of development into paragymnohymenial and eugymnohymenial according to the extent of investment of the ascogonium by overarching hyphae. Ascomata of the former had overarched ascogonia while those of the latter did not. Eugymnohymenial ascomata have only been found in Ascodesmis Tiegh., Pyronema Carus and Coprotus Korf and Kimbr. outside of several species of Ascobolus Pers. ex Fr. and a number of species of Saccobolus Bond. (van Brummelen, 1967; Corner, 1929; Claussen, 1905, 1912; Kish, 1974). Developmental studies of the apothecia of Ascobolus by Dodge (1912) revealed that cleistohymenial and gymnohymenial forms occurred within a single genus. Consequently, the type of development only had importance at the species level. Van Brummelen (1967) pointed out, however, that in Ascobolus a type of development frequently was particular for different sections of the genus, indicating greater relatedness among the species within a section. Similarly, taxonomic affinities may occur among the three nonascobolaceous eugymnohymenial taxa.

Ascodesmis, Pyronema and Coprotus have generally been placed in separate families (Table 1). The taxonomic positioning of the three genera has remained unclear. LeGal (1949) proposed that Ascodesmis was highly evolved. She based her proposal on spore sculpturing and the coprophilous nature of the fungus. Obrist (1961) concurred with LeGal and suggested that the genus Ascodesmis may be placed

Chapter III  
Table 1

Classifications of the Eugymnohymenial Genera:  
Ascodesmis, Coprotus and Pyronema

Eckblad (1968)

Thelebolaceae

Coprotus

Ascobolaceae

Ascodesmidoideae

Ascodesmis

Pyronemaceae

Pyronema

Kimbrough (1970)

Thelebolaceae

Coprotus

Ascobolaceae

Ascodesmidoideae

Ascodesmis

Pyronemataceae

Pyronema

Korf (1973)

Pyronemataceae

Ascodesmidoideae

Ascodesmis

Pyronematoideae

Pyronemateae

Pyronema

Ascophanoideae

Theleboleae

Coprotus



tentatively in the family Humariaceae tribe Humarieae sensu LeGal (1947) due to similarities in spore structure with Boudiera Cooke and Lamprospora De Not. The idea that Ascodesmis may have been highly evolved was not entirely new. Corner (1930) postulated that the smallest and simplest species including members of Pyronema, Ascodesmis and Coprotus (as Ascophanus) were the most degenerate through an evolutionary process of juvenescence. Eckblad (1968) did not agree with Corner's views but instead followed Nannfeldt's (1937) theory that pileate genera were advanced. Like Obrist, he emphasized taxonomic affinities between Ascodesmis and Boudiera. However, he disregarded the iodine-negative, nonprotruding nature of the asci of Ascodesmis and placed both genera in the Ascobolaceae.

Taxonomic positioning of Pyronema has been unclear even though morphological, ontogenetic and cytological data have been abundant (Moore and Korf, 1963). Rifai (1968) pointed out that comparable information was lacking from those genera that previously had been considered to be related to Pyronema. He stated that the true relationship of this genus could only be made after the anatomy and the ontogeny of brightly pigmented species of the Humariaceae and Thelebolaceae were understood. Eckblad (1968) noted that the presence of a reduced excipular margin, which had been neglected or overlooked by earlier workers, made it impossible to place Pyronema in a separate family.

Taxonomic affiliations of Coprotus have been similarly vague. Species which comprise this genus were treated at one time or other as belonging to Ascophanus and (or) Ryparobius (Kimbrough, Luck-Allen, and Cain, 1972). Reduction of the number of asci, increase in the number of ascospores, and uninucleate nature of the cells in paraphyses and excipulum were the main characters used to justify placing Coprotus in the Thelebolaceae (Kimbrough and Korf, 1967). Kimbrough, Luck-Allen, and Cain (1969) noted that eight-spored forms resembled members of Iodophanus, Coprobia, Peziza, Psilopezia and Pyronema. Developmental studies of Coprotus and Pyronema by Dangeard (1907) pointed to important similarities in these genera including the production of clustered ascogonia, eugymnohymenial ontogeny, and the same type of ascogenous systems. In a cytological investigation of Coprotus lacteus (Ck. and Phill.) Kimbr., Luck-Allen, and Cain, Kish (1974) stated that Coprotus demonstrated much closer morphological, developmental and cytological affinities with the Pyronemaceae sensu Eckblad (1968) than with the Thelebolaceae and suggested that it be transferred to the former family.

Studies of ascal structure have provided significant contributions to fungal systematics within the Euascomycetes (see General Introduction). Recently, a number of studies have paid close attention to the ascal wall and its associated mechanism of dehiscence (Corlett and Elliott, 1974; Beckett and Heath, 1974; van Brummelen, 1974, 1975;

Bellemère, 1975; Samuelson, 1975). Dehiscence mechanisms (described in Chapters I and II) offered useful information that has helped determine more accurately the phylogeny of certain taxa within the operculate Discomycetes.

Apical apparatuses of the eugymnohymenial members, Ascodesmis, Pyronema and Coprotus have not been critically examined. Although asci of Ascodesmis and Pyronema have been examined ultrastructurally (Moore, 1963; Reeves, 1967), little information was reported on the ascal wall. Moore (1963) disclosed in Ascodesmis that the wall, which was unitunicate, developed near maturity a "dehiscence septum." The "dehiscence septum" consisted of a broad annular thickening that apparently developed transversely in the distal region of the ascal wall. He proposed that the operculum separated along this septum.

The present study examines morphological, developmental and cytochemical features of the apical apparatus of eugymnohymenial species of Ascodesmis, Pyronema and Coprotus in order to understand more clearly their taxonomic relatedness within the operculate Discomycetes. Fresh specimens of Ascodesmis sphaerospora Obrist, Pyronema domesticum (Sow. ex Fr.) Sacc., Coprotus winteri (Marchal) Kimbr. and C. lacteus were used.

## Materials and Methods

### Collection of Materials

Young and mature apothecia of Coprotus lacteus and C. winteri were found on cow dung collected near Gainesville. A culture of Pyronema domesticum was graciously given by E. M. Landecker. A culture of Ascodesmis sphaerospora, no. 1037, was obtained from the Culture Collection of the Rancho Santa Ana Botanic Garden, courtesy of R. K. Benjamim. Apothecia of P. domesticum and A. sphaerospora were grown on PDA (Potato-Dextrose Agar) and DOA (Dung-Oatmeal Agar; Benedict and Tyler, 1962), respectively. Different developmental stages of the apothecia of C. lacteus and C. winteri were removed from the substrate and placed in thin layers of solidifying water agar. Whole squash-mounts of individual apothecia of A. sphaerospora and P. domesticum were used to determine the stage of ascal ontogeny for the majority of apothecia in a particular Petri plate.

### Procedures for Light Microscopic Observations

Whole ascocarps of C. lacteus, C. winteria, A. sphaerospora and P. domesticum were squash mounted mostly in Congo red to stain the ascal walls (Samuelson, 1975). Aniline blue and lactophenol cotton blue were also used (see Chapter I).

### Procedures for Electron Microscopic Observations

Agar blocks, each containing 6 to 12 apothecia, of C. lacteus, C. winteri and A. sphaerospora and entire

apothecia of P. domesticum were fixed in a buffered (0.2M sodium cacodylate pH 7.2) 2.0% glutaraldehyde and 2.0% paraformaldehyde solution for two hours at room temperature. All materials were rinsed, postfixed in osmium tetroxide, dehydrated, embedded, sectioned and poststained as described in Chapter I.

## Results

### The Apical Apparatus of *Pyronema domesticum*

The mature ascus is cylindric to subcylindric ranging in length from 135 to 190  $\mu\text{m}$  and in diameter from 8 to 11  $\mu\text{m}$ . The ascal wall appears uniformly thick laterally and subapically (Fig. 1). At the periphery of the tip, the wall becomes slightly thinner. The apical wall seems to stay thin and in most mounting agents is frequently concave in outline (Fig. 1). At ascal dehiscence, the operculum remains attached to the ascus (Fig. 2). The subapical wall is more constricted shortly beneath the ascostome.

At the ultrastructural level, the inner layer of the ascal wall is seen during the final stages of spore development (Figs. 3, 4, 6). Most of the deposition of the inner layer occurs in the opercular (Figs. 3, 4) region where the outer layer has a thickness of 90-100 nm. Subapically, the outer layer dramatically increases in thickness to 250-270 nm (Fig. 6). A thin band of electron-dense cytoplasm lies against the internal face of the inner layer in the apical and subapical regions of the ascus (Figs. 4, 6).



By the end of the development of the apical apparatus the opercular wall has thickened to 250-260 nm (Figs. 5, 7). Much of the wall is comprised of the inner layer with an overall thickness of 110-140 nm. The operculum is strongly demarcated after treating the thin sections with silver methenamine (Figs. 5, 7, 8). The opercular and subopercular inner layer is moderately stained (Fig. 7). The outer layer, however, is intensely stained for most of the ascal wall except at the point where it suddenly narrows in thickness at the tip (Figs. 5, 7), i.e., the operculum. In the opercular region the outer layer reacts weakly with silver methenamine offering a sharp contrast to the opercular inner layer and the subopercular outer layer. At spore liberation, the operculum partially ruptures along this differentially stained portion of the tip (Fig. 8). The operculum has a diameter of 7.4-7.8  $\mu\text{m}$ . The suboperculum is relatively short, 0.8-1.0  $\mu\text{m}$ . The inner layer tapers from 50-60 nm at its upper extremity to 20-30 nm, while the outer layer broadens from 240-270 to 280-310 nm.

#### The Apical Apparatus of *Ascodesmis sphaerospora*

Mature asci are broadly clavate to ovoid, being 70-85  $\mu\text{m}$  long and 30-35  $\mu\text{m}$  wide. The ascal wall which appears thin throughout the ascus and particularly so at the tip has no distinct features in the apical region (Fig. 9). After ascospore discharge the operculum remains partially attached to the ascus often returning to its original position

(Fig. 10). The wall region surrounding the ascostome is hyaline when treated with Congo red (Fig. 10).

Ultrastructurally, the mature ascus is frequently observed to be asymmetrical (Fig. 11). The apical wall may be eccentrically positioned in a heliotropic manner. The thickness of the ascal wall narrows from 360-390 nm at its lateral sides to 190-220 nm at the tip (Fig. 11). The inner layer of the wall is granular and barely more electron-transparent than the outer layer (Fig. 12). At the periphery of the apical wall an electron-translucent zone is observed in the inner layer (Fig. 12), delimiting the operculum. This zone, which is 280-310 nm long, most likely plays an important role in ascal dehiscence. The diameter of the operculum measures 10.5-11.1  $\mu\text{m}$ .

Staining with silver methenamine markedly enhances the appearance of the wall layering in the ascus (Figs. 13-16). The strongly stained inner layer comprises almost one-half of the thickness of the opercular wall, being 90-110 nm. The inner layer tapers to 50-60 nm at the lower extremity of the suboperculum, which is 4.8-5.4  $\mu\text{m}$  long. The less intensely stained outer layer consists of two strata (Fig. 15). The external stratum remains 60-80 nm thick throughout the opercular and distal subopercular regions of the wall, increasing slightly to 85-95 nm toward the base of the ascus. The internal stratum increases from 50-60 nm in the operculum to 250-280 nm at the lower extremity of the suboperculum. At the region of the transparent dehiscent zone observed in

Fig. 12, the outer layer is less conspicuously stained (Figs. 15, 16). At incipient spore release (Fig. 14), the outer layer is devoid of any stain at this region and the inner layer has become slightly indented. The strata of the outer layer are not discerned in the vicinity of the differentially stained dehiscent zone (Fig. 14).

#### The Apical Apparatus of *Coprotus winteri*

Mature asci are broadly cylindric reading a length of 160-200  $\mu\text{m}$  and a diameter of 40-55  $\mu\text{m}$ . Two hundred and fifty-six ascospores are formed within each ascus (Fig. 17). The wall appears quite thick for most of the ascus except at the truncate to rounded tip where the wall is considerably thinner (Fig. 18). When placed in Congo red the ascal wall is seen to be bilayered (Fig. 18). The outer layer is strongly stained in all regions but the apex.

With the aid of the electron microscope, the tips of almost mature asci (Figs. 19, 20, 21) are relatively thin-walled, 580-620 nm. Within 1.6-1.8  $\mu\text{m}$  of the apex, the wall thickens to 1250-1300 nm (Figs. 19, 30). The apical region of the ascus is filled with mitochondria, ribosomes and glycogen at this stage in development. After staining thin sections with silver methenamine, stratification of the ascal wall is accentuated (Figs. 20, 21). The wall is comprised of a single layer having three strata at this stage (Fig. 21). The external stratum remains 90-100 nm thick for the length of the ascus. The middle stratum decreases in thickness from 950-1000 nm in the region of the lateral wall

to 450-480 nm at the apex. The internal stratum is relatively thin at this time varying from 30 to 70 nm and having an irregular outline (Fig. 21).

By the end of spore wall development, an inner layer is formed in the ascus wall (Figs. 22, 24, 25). This inner layer, which is more fibrillar and electron-opaque than the outer layer, is thickest at the tip, 190-220 nm, and becomes reduced to 80-100 nm toward the base of the ascus. With silver methenamine (Figs. 23, 25) the strongly positive inner layer is sharply contrasted with the negative outer layer. The opercular boundary is demarcated by an annular swelling of the outer layer's middle stratum (arrows in Fig. 25). Furthermore, the middle and internal strata below the ring are more strongly stained. The operculum has a diameter of 9.2-9.7  $\mu\text{m}$  and a thickness of 770-820 nm. The suboperculum, which is 5.1-5.3  $\mu\text{m}$  long, thickens from 800-820 nm at its distal end to 1500-1550 nm at its proximal end. The increase is due to the thickening of the internal stratum of the outer layer and the addition of the inner layer.

#### The Apical Apparatus of *Coprotus lacteus*

During early spore development, the tips of young asci are thin-walled and round (Fig. 26). The lateral wall becomes strongly broader toward the base. At maturity, the ascus is subcylindric to clavate, 65-80 x 14-18  $\mu\text{m}$ . The tip, which becomes more blunt, remains thin-walled (Fig. 27). At dehiscence, the operculum stays fastened at one side of

the ascus (Fig. 28). The wall below the ascostome becomes constricted.

Electron microscopic observations of asci during spore wall development demonstrate the apical wall to be notably thin (Figs. 29-30), 200-220 nm. The lateral wall expands to 500-530 nm within a short distance from the tip. The wall is uniformly granular and electron-transparent at this stage in development. When staining thin sections with silver methenamine (Fig. 30), the internal portion of the lateral wall and the external boundary of the entire wall react positively.

By the end of spore development, an inner layer has formed predominantly throughout the apical and subapical regions of the ascus wall (Fig. 31). The apical wall has increased in thickness to 310-340 nm. The inner layer comprises approximately one-half of the wall at the apex, being 140-160 nm thick. The lateral wall has changed little in thickness. During the release of the ascospores, the operculum is still attached to the ascus at one side (Figs. 32, 34, 35). The outer layer remains intact at the point of attachment. The dehiscent operculum has a diameter of 7.5-7.7  $\mu\text{m}$  and an even thickness of 420-430 nm. The suboperculum is 6.0-6.2  $\mu\text{m}$  long and increases in thickness from 370-400 nm at the distal end to 510-540 nm toward the base (Fig. 32). Within the suboperculum the inner layer decreases from 130-150 nm at the upper extremity. The wall layering is greatly enhanced by the silver methenamine stain



(Fig. 33) with the inner layer being intensely stained. The outer layer is unstained except at its external boundary (Fig. 35). This thin portion of the outer layer, however, is not stained at the periphery of the operculum (arrows, Figs. 33, 35).

### Discussion

Light microscopic observations of the apical apparatus in eugymnohymenial operculate discomycetes revealed few notable characteristics. Subopercular rings, annular indentations, apical domes and apical rings described in Chapters I, II and V were not observed. Mature asci of the four representatives, however, had one major distinction. The apices were conspicuously thin-walled. In the tips of Coprotus lacteus and C. winteri the decrease of the subapical wall toward the periphery of the apex was the sharpest. Kimbrough (1970) and Kimbrough, Luck-Allen, and Cain (1972) had previously reported this character in a number of species of Coprotus. In Pyronema domesticum and Ascodesmis sphaerospora the differences in thickness between the subapical and apical regions of the ascal wall were less notable. Although the apical walls of A. sphaerospora tended to be more flaccid than the lateral wall, an operculum or opercular region was not apparent. On the other hand, the narrowing of the apical wall within a restricted area in P. domesticum and both species of Coprotus approximately delimited the operculum. The

subcylindric shape of the asci and the slight constriction at the tips in Pyronema and Coprotus aided in the delimiting of the opercular region. At dehiscence, the constriction of the lateral wall was accentuated below the ascostome. Sub-apical constrictions have not been observed in ascal tips of other operculate species (see Chapters I, II, IV, VI) in the present study. Their role in P. domesticum, C. winteri and C. lacteus most likely was involved in the discharge of the ascospores, supplying additional force during their ejection. The apical wall in P. domesticum, which was typically concave at maturity, resembled the ascal tip of Otidea leporina (Fr.) Fuckel (see Chapter II). Initial observation of the apical wall in P. domesticum gave the impression that the wall was unusually swollen. By gradually changing the focus of the oil-immersion objective lens the wall was found to be thin and sunken in this region. In O. leporina the apical swollen region was too small to determine by this method whether the wall was similarly thin and recessed. Fine structure of mature asci in P. domesticum demonstrated convex apices as in O. leporina. Light microscopic observations of the apical wall should be made cautiously. Asci that appeared flattened or swollen at their tips may have been dramatically influenced by mounting and staining procedures.

Ultrastructurally, the gross morphology of the apical apparatuses in the eugymnohymenial representatives presently studied were similar. In each species the lateral wall

decreased between 80 and 100% in thickness toward the apex. Tapering of the wall thickness was most pronounced in Pyronema domesticum, occurring in the vicinity of the zone of dehiscence. The inner layer of the four species decreased in thickness toward the base of the ascus.

Delimitation of the opercula was greatly enhanced in all species after staining with silver methenamine. Differential staining of the outer layer in P. domesticum strongly delimited the operculum from the rest of the ascal wall. In Coprotus winteri and Ascodesmis sphaerospora a ring was distinguished between the suboperculum and the operculum. At this region the outer layer, which became slightly thickened in both species, was weakly stained by silver methenamine in A. sphaerospora. By comparison, the outer layer of the ascal wall of C. winteri was mostly unreactive except for the boundaries of the strata and an area immediately below the ring. In A. sphaerospora the ring appeared to have degenerated markedly by the onset of spore release. Unfortunately, dehisced asci were not observed with the electron microscope in either species. Consequently, the exact point of rupture remained unclear. The "dehiscence septum" described by Moore (1963) for A. sphaerospora was never observed. The uneven appearance of the circumscissile thickening in his photographs indicated the possibility that they may have been grazing sections of the ascal wall. Broad transverse rings would surely have been observed by earlier workers with the light microscope.

The abrupt changes of the staining intensities of the outer layer in the region of the operculum of A. sphaerospora, C. winteri and P. domesticum indicated localized chemical differences in the ascus wall, which most likely played a critical role in ascus dehiscence.

Although the ascus wall of Coprotus lacteus did not exhibit a distinct opercular ring, the outer layer's external boundary appeared to be cytochemically differentiated at the region of the dehiscent zone. The shape of the apical apparatus was almost identical to that of C. winteri. Wall dimensions in C. winteri were roughly three times greater than those in C. lacteus. Stratification of the outer layer was observed best in C. winteri. Kimbrough and Benny (1977) described comparable layering in the multi-spored ascus of Lasiobolus monascus Kimbr. In its entirety the ascus of C. winteri was an exaggerated form of the ascus of C. lacteus. Wall components appeared to be highly accentuated in the multispored (greater than eight) species. Similar findings were described in species of Thelebolus, having different numbers of spores per ascus (see Chapter V).

Morphological and developmental features of the apical apparatus of Pyronema domesticum agreed more closely with those of C. winteri and C. lacteus than with any other operculate representative presently studied (see Chapters I, II, IV & VI). The abrupt change in thickness between the operculum and suboperculum and the distinct differential

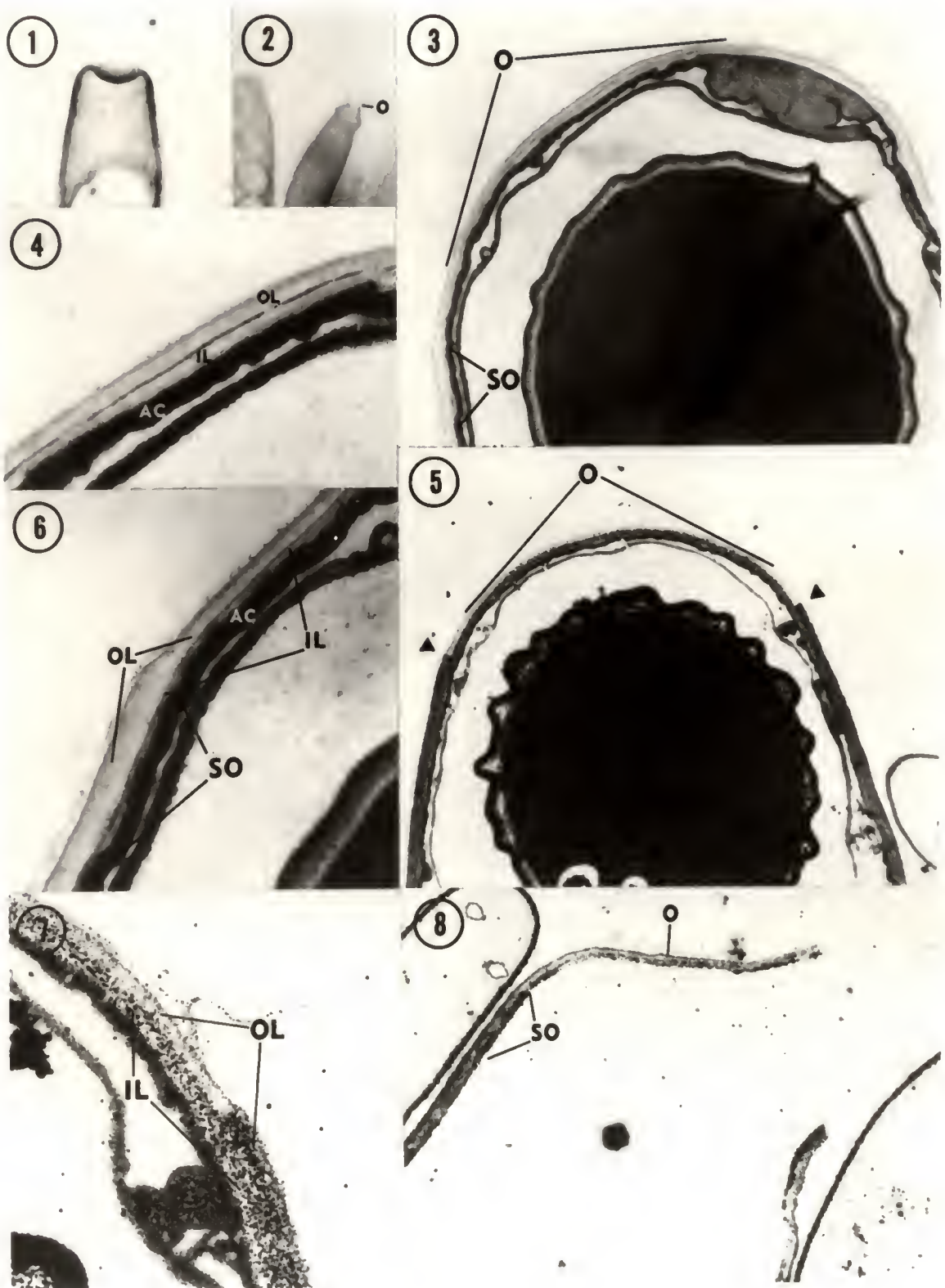
staining of the entire outer layer of the opercular wall clearly distinguished the apical apparatus of P. domesticum from the apical apparatuses of the other eugymnohymenial spp. The apical tip of A. sphaerospora with its dehiscent ring and stratified outer layer resembled that of C. winteri. Apical apparatuses found in the eugymnohymenial species of the present study were similar in form and wall thickness to Otidea leporina (see Chapter II). The present information supported the conclusions of Obrist (1963), Rifai (1968) and Kish (1974) which suggested that Ascodesmis, Pyronema and Coprotus, respectively, were most closely related to members of the Otideaceae and Aleuriaceae sensu Kimbrough (1970).

### Chapter III

#### Figures 1-8. Pyronema domesticum

- Figure 1. Concave tip of mature ascus. Stained with Congo red. X1,000.
- Figure 2. Dehiscent ascus with attached operculum (O). Stained with Congo red. X400.
- Figure 3. Development of apical apparatus during final stages of spore development. Operculum (O). Suboperculum (SO). X12,500.
- Figure 4. Opercular region of developing tip. Outer layer (OL). Inner layer (IL). Ascal cytoplasm (AC). X27,000.
- Figure 5. Mature apical apparatus with differentially stained operculum (O). Arrows point to zone of dehiscence. Stained with silver methenamine. X9,000.
- Figure 6. Subopercular region of ascal tip shows developing inner layer (IL). Outer layer (OL). Suboperculum (SO). Ascal cytoplasm (AC). X21,000.
- Figure 7. Area of dehiscent zone in mature ascus. Inner layer (IL). Outer layer (OL). Stained with silver methenamine. X26,500.
- Figure 8. Ruptured ascus with operculum (O) partially fastened to ascus. Suboperculum (SO). Stained with silver methenamine. X9,200.

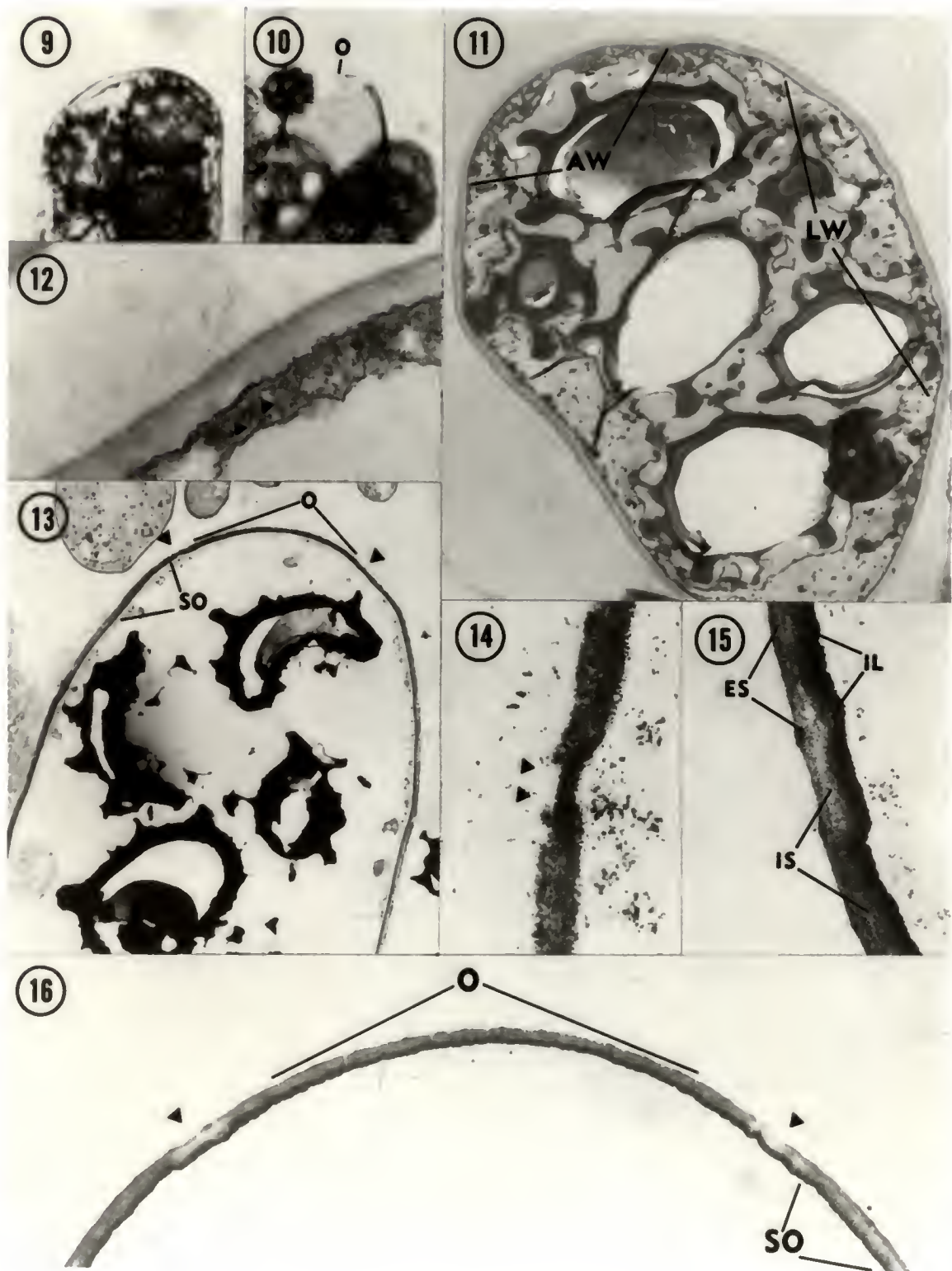




### Chapter III

#### Figures 9-16. Ascodesmis sphaerospora

- Figure 9. Apical portion of mature ascus. Stained with Congo red. X1,000.
- Figure 10. Broad operculum (O) is attached after spore release. Stained with Congo red. X5,000.
- Figure 11. Mature ascus with heliotropic apical wall (AW). Lateral wall (LW). X4,500.
- Figure 12. Electron-translucent zone (arrows) delimits operculum. X23,000.
- Figure 13. Apical portion of mature ascus stained with silver methenamine. Arrows point to zone of dehiscence. Operculum (O). Suboperculum (SO). X4,300.
- Figure 14. Zone of dehiscence (arrows) at incipient spore release. Stained with silver methenamine. X21,000.
- Figure 15. Region of zone of dehiscence showing inner layer (IL) and external stratum (ES) and internal stratum (IS) of outer layer. Stained with silver methenamine. X19,000.
- Figure 16. Ascus tip stained with silver methenamine shows layering of operculum (O) and suboperculum (SO). Arrows point to zone of dehiscence. X7,400.

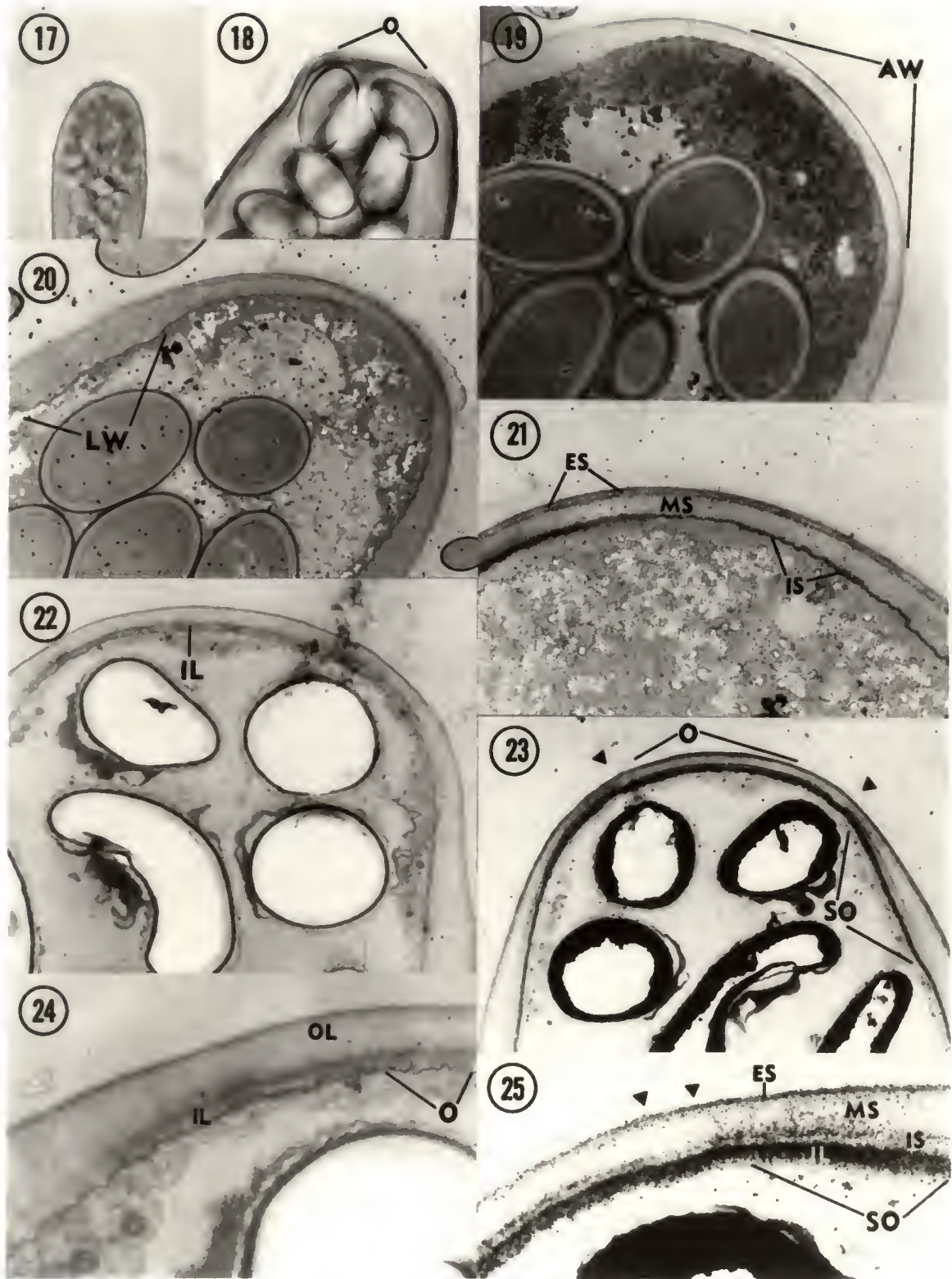


### Chapter III

#### Figures 17-25. Coprotus winterii

- Figure 17. Mature ascus containing approximately 250 ascospores. Stained with Congo red. X400.
- Figure 18. Apical portion of mature ascus shows operculum (O). Stained with Congo red. X1,500.
- Figure 19. Ascus tip near end of spore development has thin apical wall (AW). X5,000.
- Figure 20. Ascus tip near end of spore development stained with silver methenamine. Lateral wall (LW). X3,800.
- Figure 21. Apical region of almost mature ascus shows stratified wall. External stratum (ES). Middle stratum (MS). Internal stratum (IS). Stained with silver methenamine. X8,000.
- Figure 22. Mature ascus tip. Inner layer (IL). X5,000.
- Figure 23. Mature apical apparatus with operculum (O) and suboperculum (SO). Arrows point to zone of dehiscence. Stained with silver methenamine. X4,000.
- Figure 24. Region of dehiscent zone showing inner layer (IL) and outer layer. Operculum (O). X16,000.
- Figure 25. Region of dehiscent zone stained with silver methenamine. Arrows point to annular swelling (zone of dehiscence). Outer layer consists of external stratum (ES), middle stratum (MS) and internal stratum (IS). Inner layer (IL). Suboperculum (SO). X16,000.



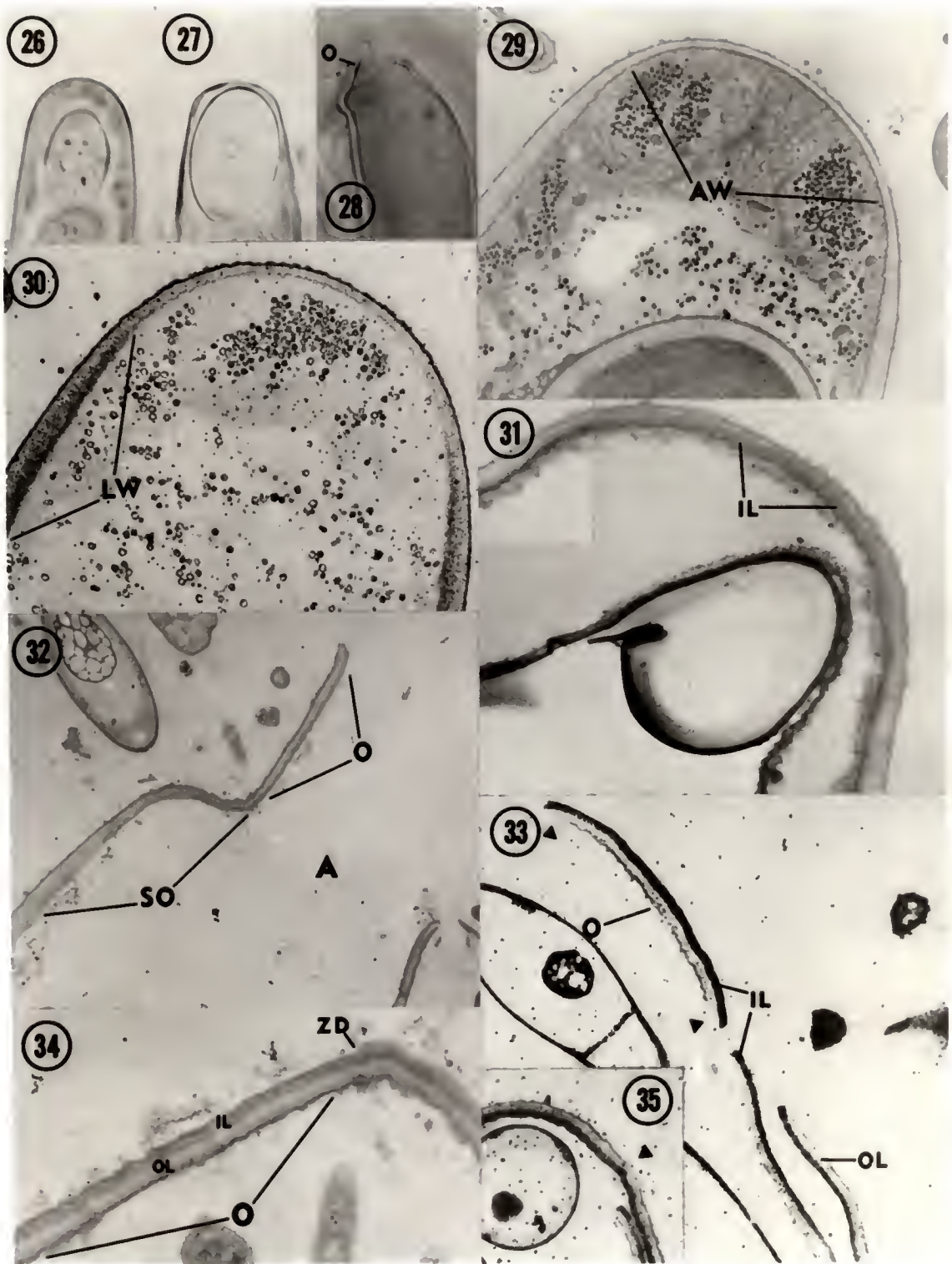


### Chapter III

#### Figures 26-35. Coprotus lacteus

- Figure 26. Ascal tip during early spore development. Stained with Congo red. X1,000.
- Figure 27. Apex of mature ascus. Stained with Congo red.
- Figure 28. Partially attached operculum (O) at spore liberation. Stained with Congo red. X1,000.
- Figure 29. Ascal tip during spore wall development. Apical wall (AW). X6,300.
- Figure 30. Ascal tip during spore wall development stained with silver methenamine. Lateral wall (LW). X8,100.
- Figure 31. Presence of inner layer (IL) in apical wall of mature ascus. X11,000.
- Figure 32. Operculum remains attached to ascus during spore release. Ascostome (A). Suboperculum (SO). X5,000.
- Figure 33. Dehisced ascus stained with silver methenamine. Arrows point to unstained area of the outer layer's external boundary. Operculum (O). Inner layer (IL). Outer layer (OL). X6,800.
- Figure 34. Region of operculum (O) and zone of dehiscence shown in Fig. 32. Inner layer (IL). Outer layer (OL). X15,000.
- Figure 35. Dehisced ascus with attached operculum. Arrow points to unstained external boundary of the outer layer at the periphery of operculum. Stained with silver methenamine. X7,100.





CHAPTER IV  
MORPHOLOGY, DEVELOPMENT AND CYTOCHEMISTRY OF THE APICAL  
APPARATUSES OF MORCHELLA ESCULENTA AND REPRESENTATIVES  
OF THE HELVELLACEAE

Introduction

Among the wide variety of representatives found within the operculate Discomycetes, members with large, stipitate variously shaped apothecia have been usually placed in the Helvellaceae and Morchellaceae sensu Rifai (1968), Kimbrough (1970) and Korf (1973). Ascospores, ascal and excipular characters have clearly distinguished the taxa, Morchella Dill. ex Fr., Verpa Sw. ex Fr. and Disciotis Boud., of the Morchellaceae from the rest of the Pezizales. Placement of genera within the Helvellaceae, however, has currently differed among authors on the basis of which characters are emphasized (Table 1). LeGal (1947) classified Discina (Fr.) Fr. and Rhizina Fr. ex Fr. in the Pezizaceae due to their apiculate or ornamented ascospores. Berthet (1964), however, demonstrated that a number of characters such as the unique tetranucleated and guttulated ascospores, the habit, habitat and pigmentation of the apothecia were features of these genera that were shared with Helvella L., Wynnella Boud. and Gyromitra Fr. Consequently, he included Discina and Rhizina in the Helvellaceae. Eckblad (1968) removed Discina, Rhizina,

Chapter IV  
Table 1

Classifications of the Helvellaceae

| LeGal (1947)         | Kimbrough (1970)    |
|----------------------|---------------------|
| Helvellaceae         | Helvellaceae        |
| Helvelleae           | <u>Underwoodia</u>  |
| <u>Helvella</u>      | <u>Helvella</u>     |
| <u>Leptopodia</u>    | <u>Discina</u>      |
| <u>Cyanthiopoda</u>  | <u>Rhizina</u>      |
| <u>Acetabula</u>     | <u>Neogyromitra</u> |
| <u>Macropodia</u>    | <u>Gyromitra</u>    |
|                      | <u>Wynnella</u>     |
| Wynnelleae           |                     |
| <u>Wynnella</u>      | Rifai (1968)        |
| Physomitreae         | Helvellaceae        |
| <u>Physomitra</u>    | <u>Discina</u>      |
|                      | <u>Rhizina</u>      |
|                      | <u>Neogyromitra</u> |
| Aleuriaceae          | <u>Gyromitra</u>    |
|                      | <u>Helvella</u>     |
| Discineae            | <u>Wynnella</u>     |
| <u>Rhizina</u>       |                     |
| <u>Gyromitra</u>     | Korf (1973)         |
| <u>Discina</u>       | Helvellaceae        |
|                      | Helvelleae          |
| Eckblad (1968)       |                     |
| Helvellaceae         | <u>Underwoodia</u>  |
| <u>Helvella</u>      | <u>Wynnella</u>     |
| <u>Wynnella</u>      | <u>Helvella</u>     |
|                      | Gyromitreae         |
| Rhizinaceae          | <u>Gyromitra</u>    |
| <u>Discina</u>       |                     |
| <u>Rhizina</u>       | Discineae           |
| <u>Gyromitra</u>     |                     |
| <u>Pseudorhizina</u> | <u>Rhizina</u>      |
|                      | <u>Discina</u>      |

Gyromitra and Pseudorhizina Jacevskij from the Helvellaceae and placed them in a separate family, Rhizinaceae, on the strength of their spore markings and unilayered excipulum. Kimbrough (1970) pointed out that the characteristic spore markings used in part by Eckblad for the erection of the Rhizinaceae were present on species of Helvella and Underwoodia Peck as well. He concluded that the characters associated with the Rhizinaceae sensu Eckblad (1968) were insufficient to warrant the formation of this family.

During the last three decades the structure of the ascus and the mechanism of spore release have received a considerable amount of attention in the Discomycetes (Bellemère, 1969, 1975; van Brummelen, 1974, 1975; Chadeffaud, 1940, 1942, 1969, 1973; Corlett and Elliot, 1974; LeGal, 1946a,b, 1953; Moore, 1963; Samuelson, 1975; Schrantz, 1970; and Wells, 1972). Chadeffaud (1942) and LeGal (1946b) were the first authors to report the phylogenetic importance of the components associated with ascus dehiscence, which both workers called the apical apparatus. Chadeffaud (1942) described six variations of the operculate apical apparatuses. The apical apparatus of Helvella sulcata Afz. was one of the variations, having differed from the others on two points. First, the apical tract was very thin and taut and did not give rise to an apical punctuation. Secondly, in a young state the ascospores were surrounded by abundant amounts of osmiophilic lipid globules. He suggested that the tract may connect the ascospores to the apical apparatus and

participate in their nutrition during the course of their development. Chadeaud (1949) later investigated the ascal characters of the Morchellaceae. Apical structures in asci of Disciotis venosa (Pers. ex Fr.) Boud. and Morchella rotunda (Fr.) Boud. were found to be consistent with the operculate type (see Chapter II). He noted the presence of lipid globules around the spores throughout ascosporeogenesis although they were less abundant than in H. sulcata.

Chapters I, II and III of the present study have shown that the apical apparatus of the operculate ascus has been useful as a systematic tool. Although cytoplasmic components of the operculate apical apparatus proposed by Chadeaud (1942) were infrequently observed with the light microscope and not detected with the electron microscope (see Chapter II), other characters such as the length and thickness of the opercular and subopercular ascal walls, the layering and staining of the wall and the presence of a subopercular ring proved useful in distinguishing the apical apparatuses of different taxa. In addition, the fine structure of ascal tips revealed certain cytoplasmic features which were peculiar to certain representatives (see Chapters I and II). The broad cylinder of glycogen in Iodophanus granulipolaris Kimbr. and the large, laminated endoplasmic reticulum in Aleuria aurantia (Oed. ex Fr.) Fuckel were not seen in apices of asci of any other representative currently studied. Chadeaud's (1942, 1949) description of lipid globules in members of the Morchellaceae and Helvellaceae



indicated similarly that this substance could be peculiar to species within the two families and thus have taxonomic significance.

In the present study I examined the morphology, development and cytochemistry of the apical apparatuses in Helvella crispa (Scop.) Fr., Morchella esculenta (L) Pers., Gyromitra infula (Schaeff. ex Fr.) Quel., Discina ancilis (Pers.) Sacc. and Rhizina undulata Fr. ex Fr. Comparisons between the species were made in order to determine degrees of similarities and differences.

### Materials and Methods

#### Collection of Material

Fresh apothecia of Helvella crispa were collected at the Devil's Millhopper five miles north of Gainesville, Florida. The material was brought to the laboratory where free-hand sections were made for light microscopic examination to determine the stage of ascal development.

Dried specimens of Morchella esculenta, Rhizina undulata and Discina ancilis were obtained from the Mycological Herbarium at the University of Florida. Dr. R. P. Korf of Cornell University generously contributed dried specimens of Gyromitra rufula. Portions of apothecia were revived at room temperature in distilled water for 6 to 12 hours in a moist chamber for light and electron microscopic observations.

### Procedure for Light Microscopic Examinations

Fresh and revived apothecia were divided into blocks, sectioned and mounted on slides as described in Chapter I. The stain, Congo red, was most frequently used to examine the ascal walls (Samuelson, 1975). Aniline blue and lactophenol cotton blue were used to observe cytoplasmic detail (see Chapter I).

### Procedures for Electron Microscopic Examinations

Five millimeter squares of fresh apothecia of H. crispa and revived apothecia of R. undulata, D. ancilis and G. rufula were fixed in buffered (0.2M sodium cacodylate, pH 7.2) 2.0% glutaraldehyde and 2.0% paraformaldehyde solution for two hours at room temperature. Five millimeter squares of M. esculenta were fixed in 1.0% permanganate solution for one hour at room temperature. All materials were postfixed in osmium tetroxide, dehydrated, embedded, sectioned and poststained as described in Chapter I.

## Results

Descriptions of the apical apparatus for each species are restricted to the operculum, zone of dehiscence and the suboperculum. Each representative is treated individually.

### The Apical Apparatus of *Helvella crispa*

Asci with young ascospores have numerous small to large lipid droplets at the tip and around each spore (Fig. 1, arrow). The wall appears uniformly thin throughout the upper half of the ascus. At maturity (Fig. 2), the ascus is

cylindric, reaching a length of 270-290  $\mu\text{m}$  and a diameter of 14-18  $\mu\text{m}$ . As the ascus approaches dehiscence, the apical wall becomes stretched and slightly flattened (Fig. 4). A hyaline ring is observed at the periphery of the tip at this time, demarcating the edge of the operculum. During spore release the operculum remains fastened to one side of the ascus (Fig. 5).

Electron microscopic observations of asci during early spore development show numerous moderately-sized lipid bodies surrounding the spores (Fig. 3). A large mass of glycogen is found in the apical region of the ascus above the lipid bodies (Fig. 3). The ascal wall is evenly electron-transparent and broadens to a small extent from 240-250 nm at the tip to 310-330 nm at the lateral wall. Toward the end of spore maturation (Fig. 6), lipid bodies are infrequently seen. The distal region of the ascus is filled mostly with the ascal vacuole, which is encased by a thin layer of ascal cytoplasm (Figs. 6, 7). The apical and lateral wall remain electron-transparent and do not change significantly in thickness (Fig. 6). At a slightly later stage in development (Fig. 7) the apical wall has increased to a thickness of 320-350 nm. Staining with silver methenamine greatly enhances the appearance of the wall layering of the mature ascus (Fig. 7). The inner layer, which is strongly positive (Figs. 8, 10), is 120-130 nm thick at the operculum and upper extremities of the sub-operculum and decreases to 40-50 nm at the lower extremity

of the suboperculum (Figs. 7, 9). The suboperculum is 6.0-6.5  $\mu\text{m}$  long and thickens from 300-320 nm at its distal region to 370-400 nm at its base. The operculum is 5.2-5.4  $\mu\text{m}$  in diameter and is bordered by an unstained area of the inner layer (Fig. 8, arrows). At ascus dehiscence the operculum stays partially attached to the suboperculum (Fig. 9). Rupture of the operculum occurs at the basal end of the unstained ring (Figs. 9, 10). The opercular inner layer and the internal region of the opercular and subopercular outer layer are distinctly less stained by silver methenamine at this time. The operculum and the upper extremities of the suboperculum have increased in thickness by 14-18% and decreased in length by 12-15%.

#### The Apical Apparatus of *Morchella esculenta*

Apices of asci during early spore formation are rounded and uniformly thin-walled (Fig. 11). The mature ascus is long and cylindric to subcylindric, 200-240 x 18-20  $\mu\text{m}$ . The apical and subapical wall is more richly stained in aniline blue than the younger tips and appears to be thicker-walled (Fig. 12).

Ultrastructurally, the ascus wall during early spore development is 290-310 nm thick at the tip and increases to 540-560 nm towards the base (Fig. 13). A thin mucilaginous coat covers the entire ascus. A small mass of glycogen is frequently observed below the tip at this stage. Lipid bodies are scattered throughout the upper portion of the ascus (Fig. 13). As the spores mature an inner layer is

formed, being prominent at the apical region of the ascus (Fig. 14). The inner layer consists of two strata in the opercular area (Figs. 14, 15). The internal stratum, 60-70 nm thick, ends in an electron-translucent ring at the periphery of the tip, delimiting the operculum (Fig. 15). The external stratum is 90-100 nm thick and more electron-dense than the outer layer and the internal stratum of the inner layer. Below the electron-translucent ring the two strata are indistinguishable (Fig. 15). The operculum has a diameter of 6.0-6.2  $\mu\text{m}$  and a thickness of 400-420 nm. The suboperculum is 4.0-4.5  $\mu\text{m}$  long and thickens from 380-400 nm at its upper extremity to 500-520 nm at its lower extremity. The inner layer tapers to a thickness of 30-40 nm toward the base of the ascus. The ascal wall is poorly stained when treated with silver methenamine except for the internal stratum of the inner layer at the operculum (Figs. 16, 18). At incipient spore release the operculum starts to rupture in the outer layer of the wall (Fig. 17).

#### The Apical Apparatus of *Rhizina undulata*

Mature asci are cylindric to subcylindric, 250-280 x 14-18  $\mu\text{m}$ . The lateral wall, which is intensely stained in Congo red, is thick-walled (Fig. 19). At ascal dehiscence the long apiculate ascospores pass through a relatively small ascostome (Fig. 21). As each spore is released from the ascus it is squeezed by the distended ascostome, which exerts additional force during ejection.



Ultrastructurally, the mature ascus has a thick lateral wall (Figs. 22, 24), 460-500 nm, which narrows in thickness to 280-300 nm at the apex. The electron-transparent inner layer is thickest at the opercular region of the ascal wall (80-90 nm) and tapers toward the base of the ascus to 40-50 nm. The outer layer consists of two strata (Figs. 23, 24). The internal stratum is electron-dense at the tip, being 100-120 nm thick, and becomes electron-transparent toward the base of the ascus (Fig. 24), having thickened to 320-340 nm. The external stratum, which is apically less electron-opaque, decreases in thickness from 80-90 nm at the tip to 60-70 nm toward the base of the ascus (Figs. 23, 24). Treating the sections with silver methenamine reveals differential staining of the apical wall (Fig. 22). The inner layer is mildly stained throughout the length of the ascus. The internal stratum of the outer layer, however, is intensely positive at the opercular region and becomes weakly stained toward the base of the ascus. The external stratum of the outer layer is the least stained portion of the ascal wall.

At spore release the operculum stays partially fastened to the ascus (Fig. 20), not breaking entirely from the outer layer at the zone of dehiscence. The suboperculum at this stage is 4.0-4.5  $\mu\text{m}$  long and increases in thickness from 310-340 nm at the distal end to 500-540 nm toward the base. The operculum is roughly 5.0-5.5  $\mu\text{m}$  long.

The Apical Apparatus of *Discina ancilis*

Mature asci are long and cylindric, 320-360 x 14-18  $\mu\text{m}$ . The lateral wall appears thick and is intensely stained by Congo red (Figs. 25, 28). In contrast, the apical wall is thinner and less richly stained (Fig. 28, arrows). At the periphery of the tip where the thickness of the wall decreases perceptibly, the wall is slightly indented, delimiting the operculum (Fig. 28). Prior to dehiscence the apiculate end of the first ascospore lies closely appressed against one side of the opercular edge, most likely aiding in the rupture of the operculum (Fig. 26).

Electron microscopic observations of apices of mature asci show the wall to be relatively thin, 300-320 nm and bilayered (Figs. 27, 29). Subapically, the wall broadens to 520-540 nm (Figs. 30, 32). The entire wall is covered with a thin mucilaginous coat which thickens from 30-40 nm at the lateral face of the ascus to 90-100 nm at the tip (Figs. 27, 29). Both layers of the wall are electron-transparent with the inner layer appearing more fibrillar (Fig. 29). When sections are poststained with silver methenamine, wall layering is clearly distinguished (Figs. 30-33). The strongly stained inner layer thickens from 80-90 nm at the lateral region of the wall to 190-200 nm at the tip (Fig. 30). Conversely, the outer layer, which is weakly positive, narrows from 400-450 to 100-120 nm (Fig. 32). The ascal wall, which is not as rigid as that observed in *Rhizina undulata*, is particularly flaccid at the tip

(Figs. 27, 30, 33). The suboperculum is roughly 7.0-9.0  $\mu\text{m}$  long. The area of the suboperculum surrounding the ascostome seems to be distinctly elastic staying constricted after dehiscence (Fig. 33).

#### The Apical Apparatus of *Gyromitra rufula*

Mature asci are cylindric, having a length of 180-210  $\mu\text{m}$  and a diameter of 12-15  $\mu\text{m}$ . Both the apices and the lateral regions of the ascus appear thin-walled (Fig. 34). During spore ejection the operculum, which is only 4-5  $\mu\text{m}$  wide, is attached to one side of the ascus (Fig. 35). The ascostome, being slightly wider than the diameter of the operculum, is 6-7  $\mu\text{m}$  across.

Ultrastructural examination of asci during early spore development reveals the presence of numerous, small lipid bodies scattered throughout the upper half of the ascus (Figs. 36, 37). The electron-transparent ascal wall tapers from 220-240 nm at its lateral sides to 110-130 nm at the tip. When staining with silver methenamine most of the wall is weakly positive at this stage in development (Fig. 37). The innermost boundary of the wall is moderately stained.

In mature asci the wall is bilayered in the apical and subapical portion of the ascus (Fig. 38). The inner layer is very electron-transparent and 80-90 nm thick for much of the apical region (Fig. 40), tapering to 40-50 nm toward the base of the ascus. The apical wall as a whole is 150-160 nm thick, being very flaccid in form. The outer layer, which is electron-dense, is thinnest in this region and more than

doubles in thickness at the lateral sides where the wall reaches its maximum thickness, 220-240 nm. After treating with silver methenamine (Fig. 39), appearance of the wall layering is particularly enhanced along the lateral wall where discernment of the inner and outer layers in sections poststained with uranyl acetate and lead citrate was difficult. The inner layer is strongly stained and the outer layer is moderately stained for the entire length of the ascal wall (Fig. 41). Changes in the staining intensity of either layer and the appearance of a zone of dehiscence do not occur. Consequently, opercular and subopercular boundaries are not delimited at any time.

### Discussion

In the present study light microscopic examination of the ascal tips demonstrated differences in wall thickness and staining intensity between the representatives. Walls in both Helvella crispa and Gyromitra rufula were uniform in thickness for most of the length of the ascus, being slightly thinner at the tip. The ascal tip of Morchella esculenta differed little from that of H. crispa and G. rufula except in having a generally thicker wall. By comparison, the thickness of the ascal wall in Discina ancilis and Rhizina undulata varied considerably from the lateral side to the apex.

Opercular delimitation was best exhibited by D. ancilis and R. undulata due to the weak staining of the ascal tip

in Congo red and the narrowing of the wall's thickness in that region. Demarcation of opercula prior to ascal dehiscence was also observed microchemically in H. crispa. The faint hyaline ring in H. crispa resembled that seen in Peziza succosa Berk. (see Chapter I). As in P. succosa the ring was thought to have been the result of either a chemical differentiation in the wall or a physical thinning of the wall. Ultrastructural evidence demonstrated that the former was the case.

Lipid bodies were detected most clearly in asci of H. crispa and to a lesser extent in G. rufula and M. esculenta. Unfortunately, the presence of lipid bodies could not be determined in D. ancilis and R. undulata since young developmental stages of asci were not examined. Nevertheless, the present findings concurred with Chadeaud's (1942, 1947) observations and strengthened the possibility that the unique occurrence of lipid bodies in developing asci of members of the Morchellaceae and Helvellaceae may be taxonomically significant.

Electron microscopic examination of apical apparatuses in the present study revealed general similarities in structure and development. In all species the inner layer of the ascal wall thickened toward the apex while the outer layer became narrower in the same direction. In each case the entire thickness of the wall increased toward the base. For R. undulata and D. ancilis the difference in thickness between the apical and lateral wall was more pronounced



than for the rest of the species. Development of the apical apparatus in H. crispa, M. esculenta and G. rufula basically followed the same pattern related for the iodine-positive ascus in Chapter I and the asci of the Otidea-Aleuria complex and eugymnohymenial species in Chapters II and III, respectively, where formation of the inner layer occurred during late ascosporogenesis.

Ultrastructurally, distinct differences among the apical apparatuses were most often observed in the layering and cytochemistry of the wall. Delimitation of opercula in intact asci was demonstrated only in H. crispa and M. esculenta where a zone or region of dehiscence was present in the inner layer. Similar dehiscent zones were detected cytochemically in apical apparatuses of eugymnohymenial representatives (see Chapter III). However, they were found to be restricted in the outer layer of the ascal wall in those species. Rupture of the operculum from the sub-operculum in H. crispa along the lower extremity of the dehiscent zone was identical to that described for the iodine-positive and eugymnohymenial species (Chapters I and III). The wall of the ascus was stratified in those species of the present study which formed the thickest walls, i.e., M. esculenta and R. undulata. In both species the stratification was most conspicuous at the tip. Wall layering in G. rufula and D. ancilis appeared to be less complex than in the rest, not having recognizable zones of dehiscence and strata within the layers. Still, it must be kept in

mind that revived material was used for the examination of the ascal tips of G. rufula and D. ancilis. Wall features such as stratification and the presence of a zone of dehiscence may have been lost in the apical apparatuses of these species.

Characters of the apical apparatuses of H. crispa and M. esculenta, including the presence of a distinct zone of dehiscence and the small variability in wall thickness of the ascal tip, suggest greater taxonomic relatedness between these species than with any other operculate group (see Chapters I, II, III, VI) and support the belief held by most authors that places the families Helvellaceae and Morchellaceae close together (Eckblad, 1968; Rifai, 1968; Korf, 1973). Except for the differences in wall thickness in G. rufula and D. ancilis, which may be in part due to the differences of the sizes of their respective asci, the apical apparatuses of G. rufula and D. ancilis are very similar, strengthening the conclusion that the two genera are closely related (LeGal, 1947; Berthet, 1964; Eckblad, 1968).

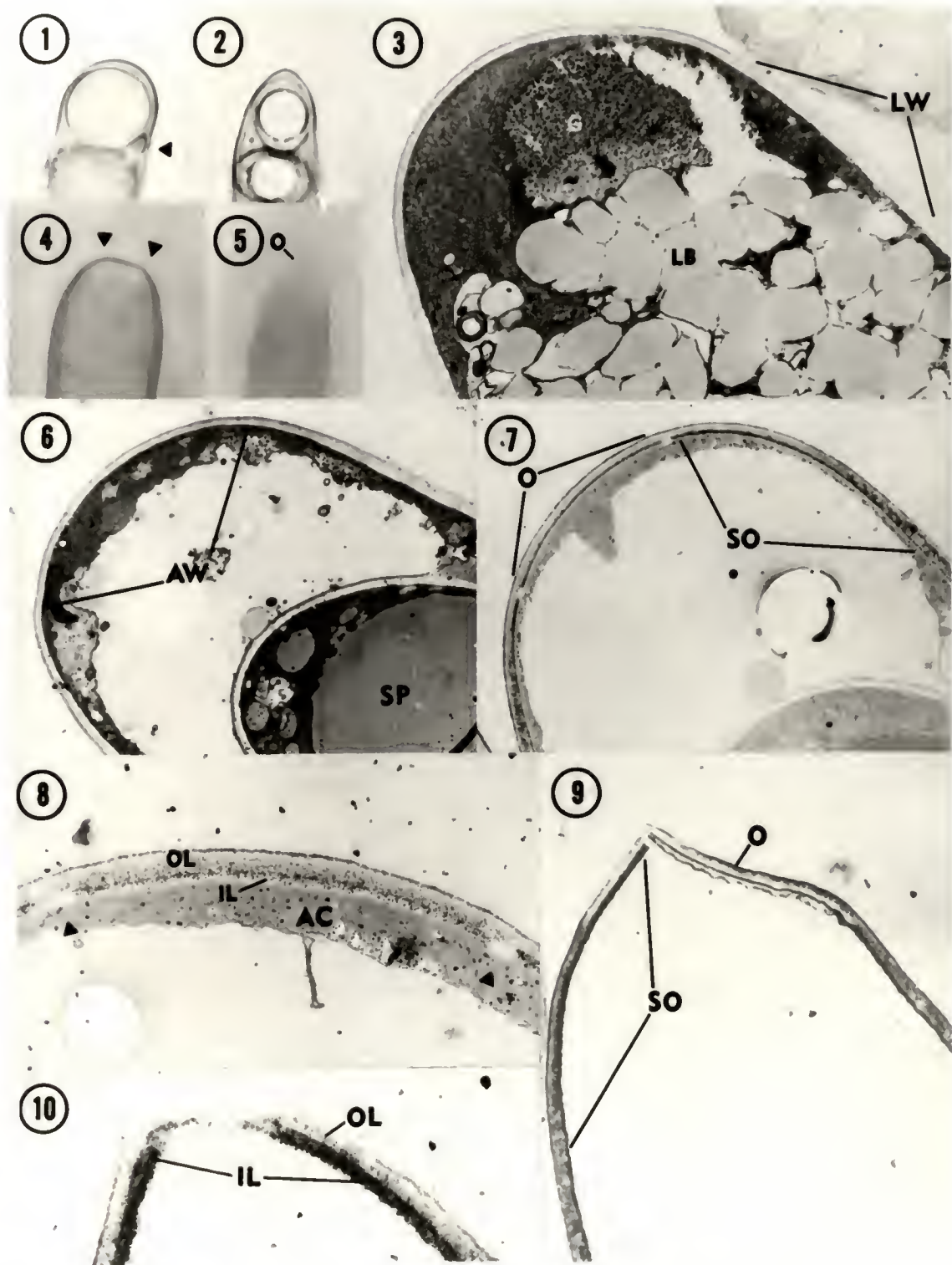
The apical apparatus of R. undulata is the most unique of the species presently studied. The unusual staining properties of the apical wall at both the light and electron microscopic level distinguished this representative from not only the other four members described in this chapter but from the rest of the operculate species discussed in

Chapters I, II, III and VI. Although Rhizina and Discina are taxonomically closely related, comparative analysis of the apical apparatuses of R. undulata and D. ancilis does not provide additional support for their affiliation.

## Chapter IV

### Figures 1-10. Helvella crispa

- Figure 1. Apical portion of ascus with young ascospores. Arrows point to lipid droplets. Stained with aniline blue. X1,000.
- Figure 2. Apical portion of mature ascus. Stained with aniline blue. X640.
- Figure 3. Ascus tip during early spore development. Lateral wall (LW). Lipid body (LB). X7,100.
- Figure 4. Before dehiscence ascus tip is stretched. Arrows point to faint, hyaline ring. Stained with Congo red. X1,000.
- Figure 5. Dehiscent ascus with operculum (O) partially attached to ascus. Stained with Congo red. X640.
- Figure 6. Ascus tip toward end of spore (SP) development. Apical wall (AW). X6,300.
- Figure 7. Apex of mature ascus stained with silver methenamine shows operculum (O) and suboperculum (SO). X6,000.
- Figure 8. Operculum of mature ascus with inner layer (IL) and outer layer (OL). Arrows point to zone of dehiscence. Ascus cytoplasm (AC). Stained with silver methenamine. X17,000.
- Figure 9. Dehiscent ascus shows differentially stained operculum (O) partially attached to suboperculum (SO). Stained with silver methenamine. X7,000.
- Figure 10. Peripheral section of dehiscent ascus shows rupture occurs at lower end of unstained zone of dehiscence. Stained with silver methenamine. X17,000

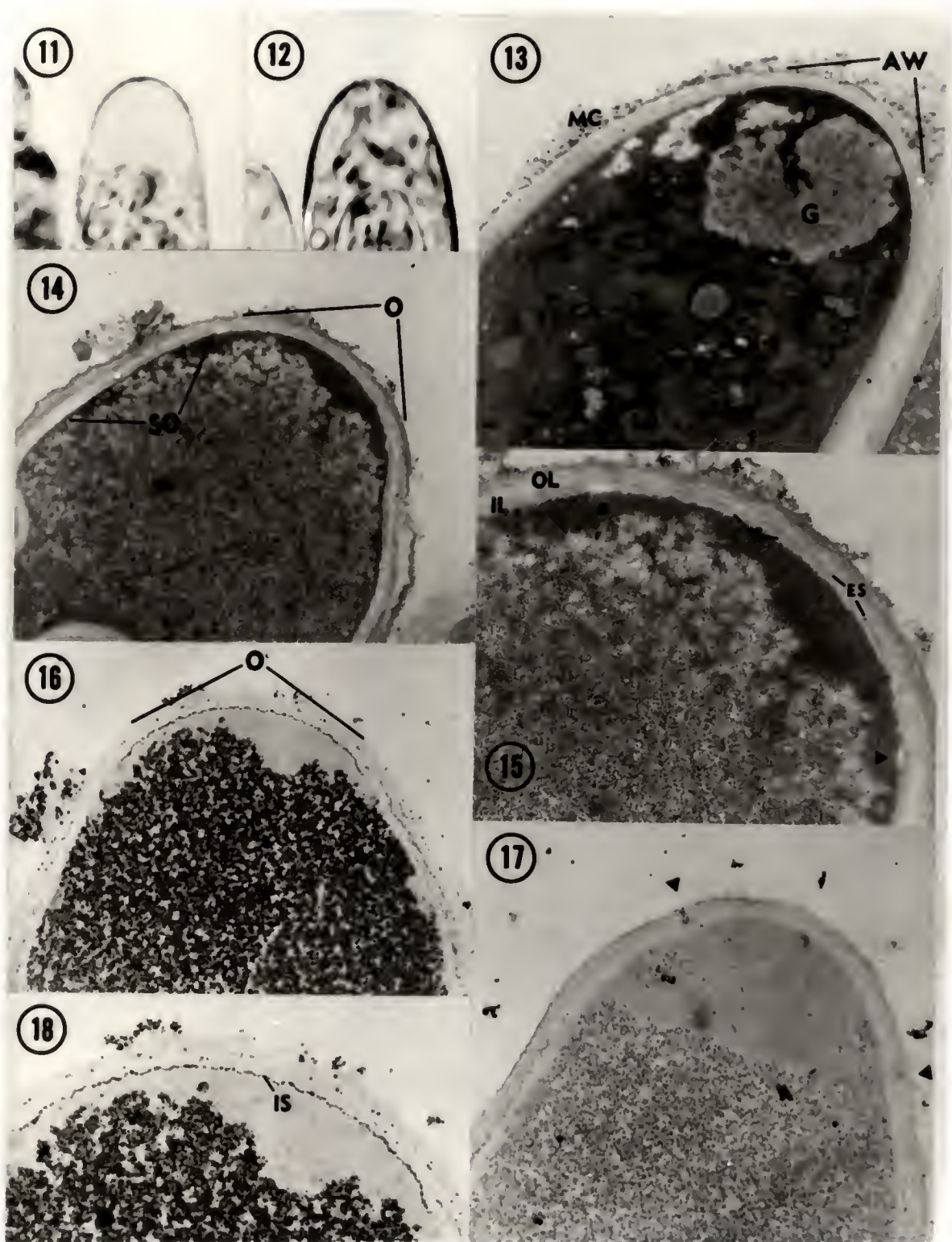




## Chapter IV

### Figures 11-18. Morchella esculenta

- Figure 11. Apical portion of ascus during early spore formation. Stained with aniline blue. X1,000.
- Figure 12. Apical portion of mature ascus. Stained with aniline blue. X1,000.
- Figure 13. Tip of ascus during early spore development. Apical wall (AW). Glycogen (G). Lipid body (LB). Mucilaginous coat (MC). X7,300.
- Figure 14. Ascus tip at later stage in spore development. Operculum (O). Suboperculum (SO). X6,900.
- Figure 15. Opercular region with arrows pointing to zone of dehiscence. Opercular inner layer (IL) consists of external stratum (ES) and internal stratum (IS). Outer layer (OL). X13,000.
- Figure 16. Ascus tip during late spore development stained with silver methenamine. Operculum (O). X6,900.
- Figure 17. Arrows point to rupturing outer layer at onset of spore release. X8,500.
- Figure 18. Opercular region shows only internal stratum (IS) of inner layer is stained by silver methenamine. X11,500.

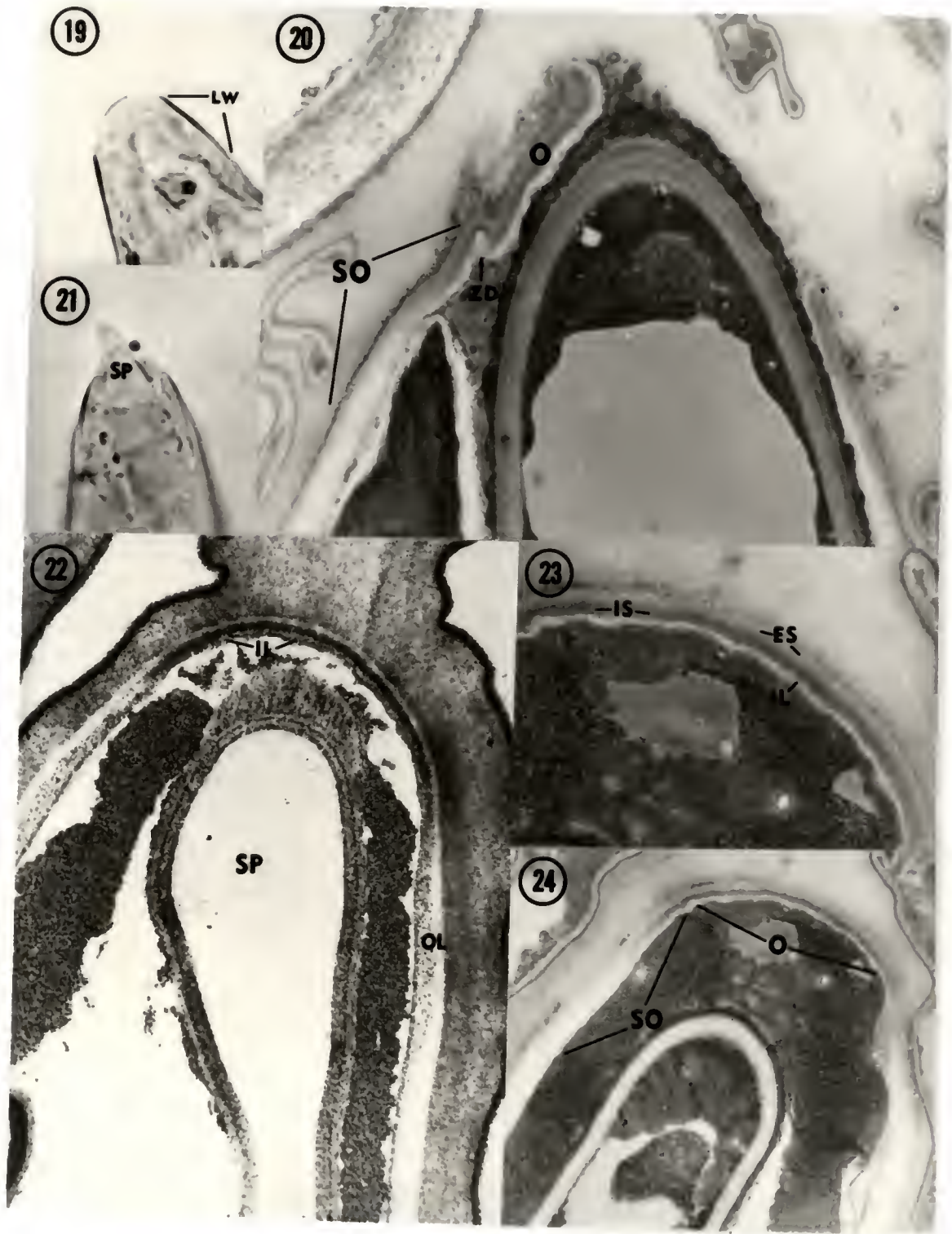


## Chapter IV

### Figures 19-24. Rhizina undulata

- Figure 19. Tip of mature ascus shows hyaline apical wall and strongly stained lateral wall (LW). Stained with Congo red. X1,000.
- Figure 20. Ascus tip at spore liberation with operculum (O) still fastened to suboperculum (SO). Zone of dehiscence (ZD). X11,000.
- Figure 21. Dehiscent ascus with spore (SP) passing through ascostome. Stained with Congo red. X1,000.
- Figure 22. Apex of mature ascus stained with silver methenamine. Inner layer (IL). Outer layer (OL). Spore (SP). X11,000.
- Figure 23. Opercular region shows outer layer is comprised of external stratum (ES) and internal stratum (IS). Inner layer (IL). X22,000.
- Figure 24. Apical region of mature ascus. Operculum (O). Suboperculum (SO). X9,500.

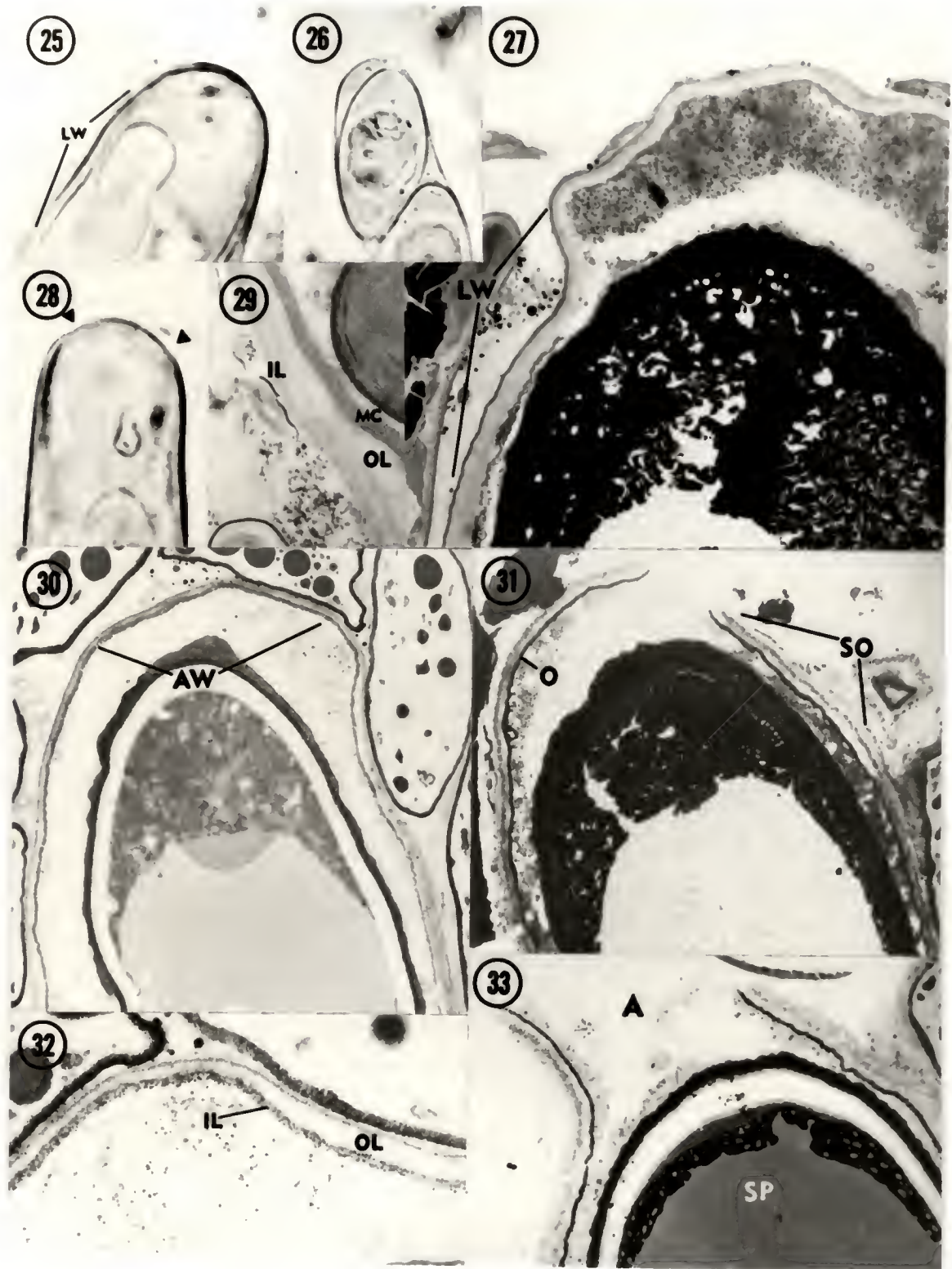




## Chapter IV

### Figures 25-33. Discina ancilis

- Figure 25. Apical portion of mature ascus with thick lateral wall (LW). X1,000.
- Figure 26. Tip of ascospore is lodged against the inner face of operculum. Stained with Congo red. X500.
- Figure 27. Tip of mature ascus. Lateral wall (LW). X8,300.
- Figure 28. Apical portion of mature ascus. Arrows point to opercular region. Stained with Congo red. X1,000.
- Figure 29. Portion of opercular wall. Inner layer (IL). Outer layer (OL). Mucilaginous coat. (MC). X28,000.
- Figure 30. Mature ascus stained with silver methenamine shows thickened inner layer at apical wall (AW). X5,000.
- Figure 31. Ruptured ascus at incipient spore release. Stained with silver methenamine. Operculum (O). Suboperculum (SO). X4,100.
- Figure 32. Subopercular portion of ascus. Inner layer (IL). Outer layer (OL). Stained with silver methenamine. X18,000.
- Figure 33. Dehiscent ascus with spore (SP) below ascostome (A). Stained with silver methenamine. X6,100.

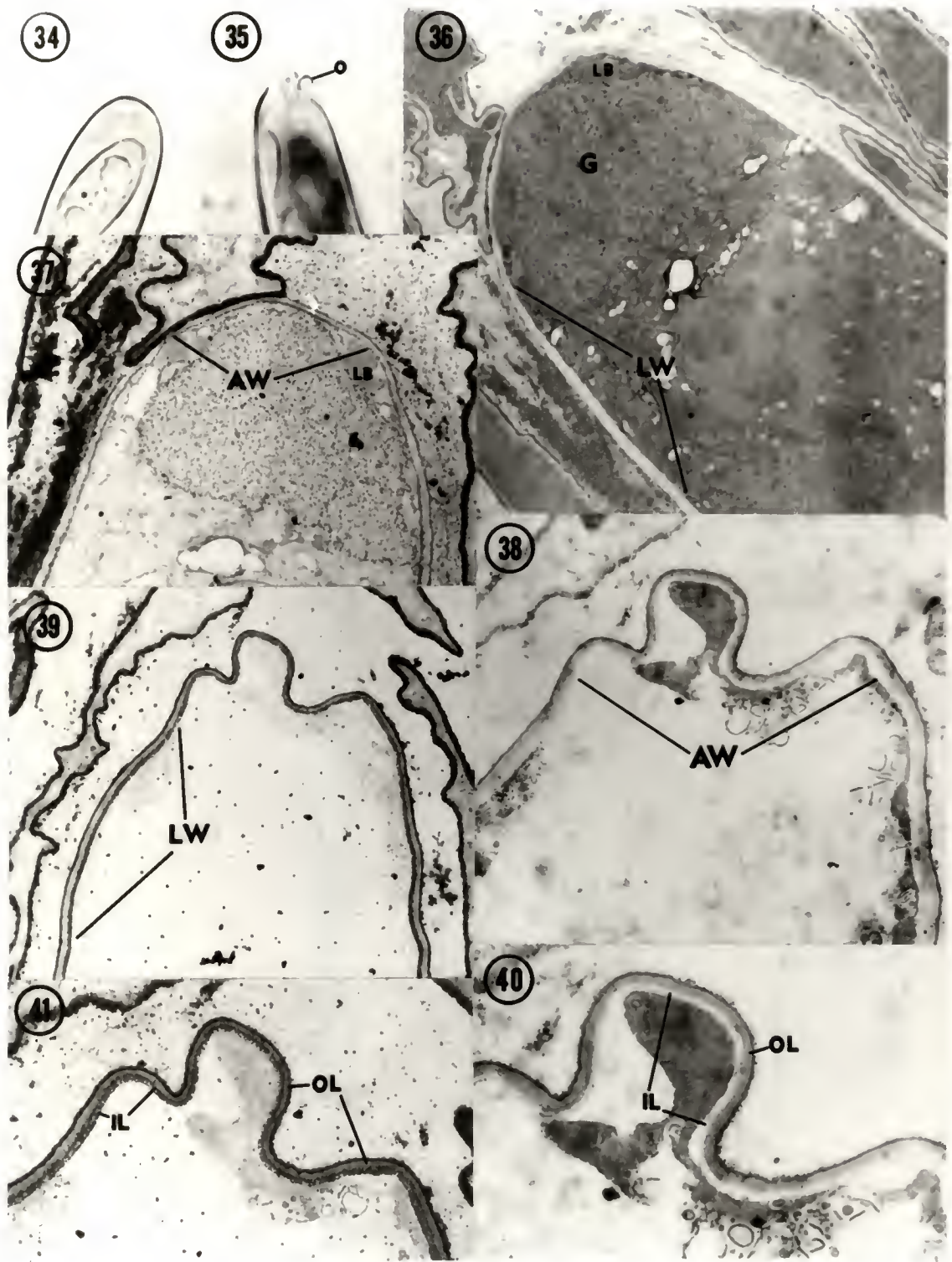




## Chapter IV

### Figures 34-41. Gyromitra rufula

- Figure 34. Apex of mature ascus. Stained with Congo red. X1,000.
- Figure 35. Operculum (O) is laterally attached during spore release. Stained with Congo red. X1,000.
- Figure 36. Apical tip during early spore development. Lateral wall (LW). Glycogen (G). Lipid body (LB). X8,000.
- Figure 37. Ascus tip during early spore development stained with silver methenamine. Apical wall (AW). Lipid body (LB). X7,700.
- Figure 38. Tip of mature ascus shows bilayered apical wall (AW). X11,000.
- Figure 39. Tip of mature ascus stained with silver methenamine. Lateral wall (LW). X8,100.
- Figure 40. Opercular region of mature tip. Inner layer (IL). Outer layer (OL). X14,500.
- Figure 41. Opercular region of mature tip stained with silver methenamine. X24,500.



CHAPTER V  
MORPHOLOGY, DEVELOPMENT AND CYTOCHEMISTRY OF THE APICAL  
APPARATUS OF THELEBOLUS

Introduction

In a study concerned with the taxonomic position of Trichobolus zukalii Heimerl within the operculate Discomycetes, Kimbrough (1966b) compared microscopical and microchemical examinations of representatives of Ryparobius Boud., Ascozonus (Renny) Boud., Thecotheus Boud., Lasiobolus Sacc., Ascophanus Boud. and Thelebolus Tode. These taxa all possessed representatives that formed asci which contained more than eight ascospores in each and are referred to here as being multispored. He demonstrated that characters which had been used at that time to separate these genera, such as the number of ascospores per ascus and the number of asci per ascocarp, were artificial and that true relationships were observed to cut across traditional generic lines. Kimbrough and Korf (1967) abandoned the name Pseudo-ascoboleae and replaced it with Theleboleae, transferring this tribe to the Pezizaceae. They incorporated within this tribe those taxa which had (1) eight and multispored, non-amyloid, operculate or irregularly dehiscent asci which generally protruded at maturity; (2) hyaline ascospores;

(3) minute, glabrose to setose apothecia; (4) coprophilous habitat. The manner in which the spores were released proved to be the most distinctive feature and was implemented to help characterize certain genera. Ascal dehiscence occurred by either an irregular tear, bilabiate split, apical plug, evanescence or circumscissile rupture. Kimbrough (1966b) relied on the staining properties of the ascal wall and the associated mechanism of dehiscence to form more natural groupings. As a result four new genera, Coprobolus, Iodophanus, Trichobolus and Coprotus, were erected. Two old genera, Ryparobius and Ascophanus were invalidated and the genera Thelebolus and Lasiobolus were extended (see Table 1). The type of apical apparatus for each genus was believed to be consistent for all of its species. The work performed by Kimbrough (1966a, b; Kimbrough and Korf, 1967; Cain and Kimbrough, 1969) on the Pseudoascobolaceae and related taxa was a major contribution to the taxonomy of this group of operculate Discomycetes.

The different types of apothecial development, cleistohymenial in Thelebolus, Trichobolus and Lasiobolus and eugymnohymenial in Coprotus and possibly Ascozonus, and the wide range of apical apparatus types found in the Thelebolaceae (sensu Eckblad, 1963; Kimbrough, 1970) have indicated evolutionary convergence along several more lines. Of the seven genera presently included in the Thelebolaceae sensu Rifai (1968) only Lasiobolus and Coprotus have been shown to be truly operculate. Recent developmental studies

Chapter V  
Table 1

Classifications of the Thelebolaceae

Boudier (1907)

Ascobolaceae

Pseudoascoboleae

Boudierella  
Cubonia  
Thecotheus  
Ascophanus  
Lasiobolus  
Ryparobius  
Ascozonus  
Thelebolus  
Aphanoascus

Eckblad (1968)

Thelebolaceae

Thelebolus  
Caccobius  
Coprobolus  
Ascozonus  
Coprotus  
Thecotheus  
Trichobolus  
Lasiobolus  
Leporina

Dennis (1968)

Ascobolaceae

Pseudoascoboleae

Lasiobolus  
Iodophanus  
Thecotheus  
Ascophanus  
Ascodesmis  
Ascozonus  
Pyronema  
Ryparobius  
Sphaeridiobolus

Thelebolaceae

Thelebolus

Kimbrough (1970)

Thelebolaceae

Ascozonus  
Caccobius  
Coprobolus  
Coprotus  
Lasiobolus  
Thelebolus  
Trichobolus



(Conway, 1975; Kish, 1974) proposed the transfer of both taxa to the Pyronemaceae sensu Eckblad. Due to a number of significant properties, the apical apparatuses of Lasiobolus and Coprotus have been discussed separately in Chapters II and III. Two members, Ascozonus and Coprobolus, discharge their spores through vertical slits at the ascal apices. Van Brummelen (1974) pointed out the presence of an apical disc in Ascozonus woolhopensis (Berk. and Br. apud Renny) Hansen prior to and after dehiscence. This evidence, plus information concerning the cytochemistry, wall layering, and developmental sequence obtained in the current investigation and by van Brummelen has resulted in categorizing the apical apparatus of A. woolhopensis with those of the Otidea-Aleuria complex (see Chapter II). The apical apparatus in Caccobius strongly differed from that of all other representatives of the Pezizales in that it possessed a plug which tore irregularly at the time of spore liberation. This type of apical apparatus resembled the inoperculate form described by Bellemère (1969, 1975). Unfortunately, specimens of Caccobius and Coprobolus were not available for the present study. Examination of their apical apparatus will have to await further study. The remaining two representatives, Trichobolus and Thelebolus, have been reported to release their spores through an irregular tearing of the apical wall (Kimbrough, 1966a, b). The apical apparatus in Trichobolus has been described separately due to its peculiar wall structure (see Chapter VI).

Thelebolus was emended by Kimbrough (Kimbrough and Korf, 1967) to include species formerly placed in Ascophanus, Ryparobius and Ascozonus. Properties of the ascal wall were greatly emphasized. When stained with Congo red the presence of a slight to prominent ring below a hyaline apex was common for all species. Irregularly dehiscent tips, cleistothecial ascocarps and thick-walled, hyaline ascospores without de Bary bubbles were other ubiquitous characters.

Cooke and Barr (1964) had proposed previously the transfer of Thelebolus stercoreus Tode per Fr. from within its own family Thelebolaceae to the Erysiphales. They based their decision on the shape of the ascus, the lateral and apical thickness of its wall, the mechanism of ascal dehiscence, the comparative development of the ascocarp and the production of a single ascus. Kimbrough (1966b) pointed out that if they had studied other members of the Pseudoascobolaceae their conclusions and taxonomic decision would have been different. Characters including the more thinly walled apex and single ascus per ascocarp were superficial and coincidental.

Delimitation of species within Thelebolus was found to be most difficult (Kimbrough and Korf, 1967). The repeated association of forms and broad overlapping of characters among previously described species observed by earlier workers (Boudier, 1869; Karsten, 1871; Barker, 1903, 1904) and by Kimbrough and Korf suggested that the ascus number, ascospore number and ascocarp size and color may be variable

in Thelebolus. Wicklow and Malloch (1971) compared the growth and development of Thelebolus isolates over a range of temperatures partly in order to evaluate the cultural stability of the previously mentioned morphological characteristics and to note whether descriptive features used in characterizing the genus could be increased. They found that the spore number was constant within any given strain and that the low temperature optima for apothecial growth and development were the same for all isolates, which explained in part why specimens have been described to contain varying numbers of spores per ascus.

The taxonomic position of Thelebolus has remained unresolved. The cylindric to spherical asci, unusual ascal tips and associated mechanism of dehiscence are characters that distinguish this genus from the rest of the operculate Discomycetes. The present study was designed to examine the morphology, development and cytochemistry of the ascal wall and its apical apparatus in Thelebolus and to understand more clearly the taxonomic placement of this genus in the Euascomycetes. Four representatives, each having a different spore number per ascus, were used. They were Thelebolus stercoreus, T. crustaceus (Fuckel) Kimbr., Thelebolus polysporus (Karst.) Otani and Kanzawa and T. microsporus (Berk. and Br.) Kimbr.

## Materials and Methods

### Collections of Material

Thelebolus stercoreus was isolated from cow dung collected near Gainesville, Florida, by K. Conway. Thelebolus polysporus and T. crustaceus were isolated by J. Kimbrough from rodent dung collected at Bodeza, Arizona, and rabbit dung at Mohave, California, respectively. The eight-spored species of Thelebolus, no. F34.68, was obtained from D. Wicklow of the University of Pittsburgh. Apothecia of each species were grown on DOA (Dung-Oatmeal Agar; Benedict and Tyler, 1962). Whole squash mounts of individual apothecia were used to determine the stage of ascal ontogeny within a circular region of the Petri plate.

### Procedure for Light Microscopic Observations

Whole ascocarps of T. stercoreus, T. crustaceus, T. eight-spored species and T. polysporus were squash-mounted in either Congo red (Samuelson, 1975) or acid fuchsin (Kimbrough, 1966a) to stain the layers of the ascal wall. Plastic embedded material was sectioned, mounted and stained in the manner described in Chapter I.

### Procedures for Electron Microscopic Observations

Five-millimeter blocks of agar, each containing numerous apothecia, of T. stercoreus, T. crustaceus and T. polysporus were fixed in a buffered (0.3M sodium cacodylate pH 7.2) 2.0% glutaraldehyde and 2.0% paraformaldehyde solution for 12 hours at room temperature. Apothecia of T.

microsporus were fixed in the same buffered glutaraldehyde-paraformaldehyde solution for two hours at room temperature. All materials were rinsed, postfixed in osmium tetroxide, dehydrated, embedded and poststained as described in Chapter I.

### Results

The Thelebolus ascus does not possess a true operculum and suboperculum. Ascal dehiscence occurs by an irregular rupture of the wall at the tip. This region of the ascal wall is called the apical dome and is subtended by a ring which was first reported by Kimbrough (1966b) after staining the wall with either Congo red or acid fuchsin. Descriptions of the apical apparatus for all species will be restricted mostly to these regions of the ascus. Each representative will be treated individually.

#### The Apical Apparatus of Thelebolus microsporus

Young asci at the four- and eight-nucleated stage are small, subcylindric to clavate, 30-40 x 5-7  $\mu\text{m}$ . The ascal wall appears to be uniformly thin throughout. At maturity the ascus reaches a length of 50-65  $\mu\text{m}$  and a width of 7-10  $\mu\text{m}$  (Fig. 1). The apical region is thick-walled, forming an apical dome (Fig. 2). When asci are placed in Congo red, an area below the tip is strongly stained delimiting the apical ring (Fig. 3).

Electron microscopic observations of a four-nucleated ascus show the wall of the ascus to be fairly even in



thickness for most of the length of the ascus (Fig. 4). The lateral wall is 250-260 nm thick and narrows slightly to 170-180 nm at the apex. The irregular outline of the internal region of the wall indicates some growth at this time in ascosporogenesis (Figs. 4, 5). In the distal portion of the ascus numerous small to moderately large vesicles are formed centrally. Mitochondria, ribosomes and endoplasmic reticulum surround the vesicles. Glycogen occurs in small packets throughout the ascus. When staining with silver methenamine at this stage in development two strata are distinguished (Fig. 5). The external stratum is intensely positive and 90-100 nm thick. In contrast, the internal stratum is weakly stained and increases from 70-90 nm at the tip to 140-160 nm toward the base. Near the end of ascospore development (Fig. 6), the apical and lateral walls have increased in thickness to 260-320 nm. The sharply irregular outline of the interior of the wall suggests pronounced wall deposition at this time. Numerous small vesicles fill much of the ascus tip and surround each spore. At a later stage in development (Figs. 7, 9), the apical wall has thickened to 320-380 nm. The increase is mostly due to the formation of an inner layer which is fibrillar and to a small extent electron-opaque (Fig. 9). The ascus is highly vesiculated and contains small amounts of cytoplasm appressed against the internal surface of the wall. By spore maturity the apical wall has reached a thickness of 450-480 nm which extends 2.4-3.0  $\mu\text{m}$  down the sides of the ascus, delimiting

the apical dome (Fig. 8). Below this point the wall tapers to a thickness of 320-350 nm. Treatment with silver methenamine, which enhances the appearance of the wall layering, shows the inner layer to be strongly stained, especially within the dome (Fig. 8). Within the apical dome the inner layer is 150-160 nm thick and narrows to 60-70 nm toward the base of the ascus. The internal stratum of the outer layer is 180-200 nm thick for the entire length of the ascus and is faintly stained at a localized region on the apical dome (arrows in Fig. 8). This area most likely represents the Congo red-positive apical ring in Fig. 3.

#### The Apical Apparatus of *Thelebolus crustaceus*

Young, uninucleated asci are globose to ovoid, being 25-45  $\mu\text{m}$  long and 15-25  $\mu\text{m}$  wide. The wall appears thin and lacks distinct features at this stage in development. Mature asci contain 64 ascospores which are broadly clavate to ellipsoid, 25-30 x 85-100  $\mu\text{m}$  (Fig. 10). The ascal wall is markedly thick throughout the ascus. The wall is faintly thinner at the ascal tip and remains hyaline when placed in Congo red except for a thin external layer (Fig. 11). Below the hyaline apex, which is referred to as the apical dome, an intensely stained ring, 4-5  $\mu\text{m}$  long, is detected. The apical ring appears to be a part of an internal stratum of the outer layer which extends most of the length of the ascus and ends beneath the apical dome. A thin external stratum, which is less positive in Congo red, covers the entire ascus. Most of the ascal wall's thickness is due to

the presence of a thick inner layer (Figs. 10, 11), which is not stained in Congo red.

Ultrastructurally, the uninucleated ascus is evenly thin-walled, 320-360 nm (Figs. 12, 13, 14). When stained with lead citrate and uranyl acetate, the primary wall of the ascus is uniformly electron-transparent (Fig. 13). However, when staining with silver methenamine, two strata are distinguished (Figs. 12, 14). The strongly positive external stratum is 210-230 nm thick. The weakly stained internal stratum is jagged in outline and frequently has small to large lomasomes attached to it (Figs. 13, 14). Numerous, small packets of glycogen are scattered throughout the vesiculated cytoplasm of the ascus (Figs. 12, 13, 14).

During early ascosporogenesis (Fig. 15), the ascus wall has thickened conspicuously to 2100-2200 nm. The additional thickness is primarily due to the formation of more inner layer. Sections that have been treated with silver methenamine reveal the presence of banding within the inner layer, which is best detected in peripheral sections (Figs. 15, 16). The ascus is filled centrally with vesicles at this stage in development (Fig. 15). The wall of the ascus is fully developed by the time of spore wall formation (Fig. 17). The apex and lateral walls reach 2600-2650 nm in thickness. When poststaining with uranyl acetate and lead citrate the wall is uniformly electron-transparent throughout the ascus (Fig. 19). However, after staining with silver methenamine the apical dome and subtending ring

are distinguished (Figs. 17, 18). The apical dome has a diameter of 15.0-16.5  $\mu\text{m}$  and is primarily composed of a thickened inner layer which is weakly stained by silver methenamine except for its internal region (Fig. 18). The positively stained apical ring, an extension of the outer layer's external stratum, is 3.70-4.20  $\mu\text{m}$  long and 1.55-1.60  $\mu\text{m}$  thick. At the dome the internal stratum tapers abruptly (Fig. 18, AR). The external stratum remains evenly thick, 80-100 nm, throughout the entire wall including the apical dome.

#### The Apical Apparatus of Thelebolus polysporus

Uninucleated asci are globose, 10-25  $\mu\text{m}$  in diameter, and thin-walled. Fine structure of the uninucleated ascus demonstrates an electron-translucent primary wall, 260-300 nm thick (Fig. 20). As in T. crustaceus, vesicles are scattered throughout the cytoplasm, being concentrated toward the internal face of the wall. The diploid nucleus is centrally oriented in the ascus at this time (Fig. 20).

During early mitotic divisions the ascus expands mostly lengthwise, being 50-55  $\mu\text{m}$  long and 25-30  $\mu\text{m}$  wide (Fig. 22). The apical region is filled with small vesicles which become progressively larger toward the base. Packets of glycogen are interspersed among the vesicles (Figs. 22, 23). The thickness of the ascal wall has markedly increased laterally and apically (Figs. 22, 25). However, the wall remains thin, 280-310 nm, at the base of the ascus (Fig. 23). An ascal pore, which is found in this vicinity (Figs. 22,

23), apparently connects the cytoplasm of the ascus to the adjoining stalk cell during this stage of development. When stained with silver methenamine the appearance of the wall layering is sharply enhanced (Fig. 21). Most of the increased thickness of the wall is due to the formation of a uniform inner layer which reacts weakly in silver methenamine. The addition of the inner layer occurs along the internal face of the lateral wall and at the immediate region of the tip where the expansion of the inner layer is greatest, being 2.9-3.0  $\mu\text{m}$  thick (Fig. 25). Immediately below the swollen tip, the inner layer narrows to 50-100 nm for a length of 3.6-4.0  $\mu\text{m}$  before broadening to 2.2-2.4  $\mu\text{m}$  toward the base of the ascus (Figs. 21, 25). The outer layer of the ascus walls consists of two strata (Fig. 21). The intensely stained external stratum is 200-240 nm thick for the entire length of the ascus. In contrast, the internal stratum, which is less strongly stained in silver methenamine, increases in thickness from 550-600 nm at the lateral wall to 1.2-1.3  $\mu\text{m}$  at the region of the wall that subtends the swollen inner layer at the apex (Figs. 21, 25). The internal stratum then abruptly tapers in thickness below the swollen tip. In this manner the apical dome and ring are distinguished.

At a slightly later time in development, the wall thickens significantly at the apical and subapical regions and less markedly at the lateral regions (Figs. 26, 34). One-micron sections stained in toluidine blue (Fig. 26)



exhibit a stratified inner layer (labelled 1&2) as well as an outer layer (labelled 3&4). After treatment with silver methenamine the four strata are readily distinguished (Fig. 24). The increase in thickness of the wall, which is 3.6-3.7  $\mu\text{m}$  throughout the ascus, is primarily due to the addition of the internal stratum of the inner layer. Furthermore, the internal stratum of the inner layer is the only area of the wall where microfibrils of the wall are distinctly banded (Fig. 24).

The mature ascus is broadly clavate to ovoid, 30-45 x 80-120  $\mu\text{m}$  and contains approximately 250 ascospores (Fig. 27). When placed in Congo red, the wall at the tip remains hyaline except for an extremely thin external stratum (Fig. 29). The wall is broadly stained below the clear dome for a short distance, 4-6  $\mu\text{m}$ , delimiting the apical ring (Figs. 27, 29). The rest of the ascus wall is stained only in the outer layer, which is moderately thin. Most of the wall consists of a thick, hyaline inner layer.

Ultrastructurally, the wall of the mature ascus is changed very little in form and thickness from that observed during early ascosporogenesis (Figs. 24, 26, 28, 34). Wall layering in the apical and lateral regions is weakly distinguished in sections that have been stained with uranyl acetate and lead citrate (Figs. 28, 30). Although the internal and external strata of the outer layer are to some extent demarcated at the apical dome (Fig. 30), stratification of the outer and inner layers is most clearly observed

at the base of the ascus where the wall narrows sharply (Figs. 28, 32). Several nuclei are present in this vicinity even though the development of the ascospores is nearly completed. The apical dome is conspicuously demarcated after staining with silver methenamine (Fig. 31). The dome has a diameter of 16.5-17.0  $\mu\text{m}$  and a thickness of 3.1-3.2  $\mu\text{m}$ . The strongly stained subtending ring is 4.0-5.5  $\mu\text{m}$  long and 2.9-3.1  $\mu\text{m}$  thick. The lateral wall is 3.2-3.3  $\mu\text{m}$  thick and narrows greatly to 1.0-1.1  $\mu\text{m}$  at the base of the ascus.

Prior to ascal dehiscence, the apical and subapical regions of the wall are distinctly stretched. The numerous ascospores are tightly lodged against the distal portion of the ascal wall. At spore release, the apical dome is distended beyond recognition, rupturing irregularly as the spores are ejected from the ascus (Fig. 35). The apical ring appears to stay intact after the spores have left the ascus. Ultrastructural examination of incipient spore release reveals the manner of dehiscence (Fig. 33). Initially, the outer layer splits roughly in the middle of the apical dome while at the same time the inner layer pushes through this opening. The inner layer becomes greatly stretched and ruptures often in the vicinity of the apical ring. The internal stratum of the outer layer at the ring does not change significantly in thickness and length before, during and after spore release, indicating that this portion of the ascal wall is structurally rigid. In contrast, the inner layer throughout the ascus appears to be very flexible

and undergoes great changes in size and form during spore ejection.

The Apical Apparatus of Thelebolus stercoreus

The young, multinucleated ascus is globose and very thick-walled, up to 8-12  $\mu\text{m}$ . In Congo red, face-on view of the apex of the young ascus demonstrates a ring, 24-28  $\mu\text{m}$  in diameter (Fig. 36). As the ascus continues to grow becoming subglobose to ovoid in shape, the wall decreases in thickness, coming 8-9  $\mu\text{m}$  at the lateral region and 7-8  $\mu\text{m}$  apically (Fig. 37). A hyaline apical dome, 42-44  $\mu\text{m}$  in diameter, is observed after staining with Congo red (Fig. 39). The wall below the dome is intensely stained for a length of 12-15  $\mu\text{m}$ , delimiting the apical ring. At the base of the ascus a wide pore extends approximately two-thirds across the wall (Fig. 43). At this point, the pore becomes very narrow as it passes through the outer extremity of the wall.

The mature ascus reaches a length of 160-250  $\mu\text{m}$  and a width of 100-150  $\mu\text{m}$  (Fig. 38). The wall is significantly thinner at the tip, being 5.5-6.0  $\mu\text{m}$  in the region of the dome (Fig. 40). At the same time, the apical dome is expanded to a diameter of 48-52  $\mu\text{m}$ . In Congo red, the apical ring is more distinct at this stage in development (Fig. 40). As in T. crustaceus and T. polysporus, the broad, positive reaction in the wall appears to be restricted to an internal stratum of the outer layer which becomes attenuated toward the base and narrows abruptly below the hyaline dome. A

thin external stratum is mildly stained in the apical dome and the remainder of the ascus. Most of the ascus is comprised of a thick inner layer, which is unstained in Congo red as shown in Fig. 42 where the outer layer has been peeled from the lateral wall. In acid fuchsin, layering within the inner layer is detected (Fig. 41). A broad external stratum is found throughout the ascal wall except at the apical ring where it is interrupted by the unstained internal stratum of the outer layer. Inside the inner layer's external stratum, an internal stratum, which is faintly stained by the acid fuchsin, is found after close examination (Fig. 41, arrow). When external pressure is applied to the mature ascus, the wall is markedly stretched at the tip of the ascus (Fig. 44). The apical dome increases 10-15% in diameter and decreases more than 50% in thickness. The rest of the wall appears to have changed little in thickness.

Ultrastructural examination of asci during early spore-wall development demonstrates the bilayered nature of the lateral wall, with an electron-opaque, 340-380 nm thick outer layer and an electron-translucent, 7.8-7.9  $\mu\text{m}$  thick inner layer (Fig. 50). Large masses of glycogen are located at the periphery of the ascal cytoplasm. At the base of the ascus, the inner layer is separated more clearly into internal and external strata (Fig. 52). In this vicinity of the wall a large pore extends through most of the inner layer (Fig. 53). Several nuclei are seen in the area of the pore.

The mature ascus has a very prominent laminated wall for its entire length (Fig. 45). The wall at the base and sides of the ascus is 7.5-7.8  $\mu\text{m}$  thick, consisting mostly of a broad inner layer. Banding occurs in the internal region of the inner layer for most of the ascus wall (Fig. 51). At the junction of the apical ring and apical dome the wall tapers considerably to a thickness of 5.5-5.6  $\mu\text{m}$  (Figs. 45, 48). The apical ring is primarily composed of a localized electron-opaque thickening of the internal stratum of the outer layer (Fig. 48). When stained in silver methenamine the internal stratum at the ring and throughout the rest of the ascus is mildly positive (Fig. 49). By comparison, the external stratum of the outer layer is intensely positive. The apical dome consists mostly of a thick, electron-translucent inner layer which appears stratified when stained with silver methenamine (Fig. 47). The internal stratum of the inner layer, 860-900 nm thick, is strongly stained at the dome. Within the external stratum of the inner layer a distinct split is frequently seen near the outer layer throughout the dome (Figs. 46, 47). During dehiscence the schism may aid in the release of the spores as the inner layer becomes distally distended.

#### Discussion

Using fresh material placed in Congo red of the four species presently studied similar cytochemical and morphological characters of the apical wall of the ascus were



demonstrated. The presence of thick-walled apical domes, which were subtended by distinctly stained rings, was common features in the apical apparatus of each representative. These findings were in complete accordance with those made by Kimbrough (1966b; Kimbrough and Korf, 1967).

Two trends were observed in the present study with regard to the number of spores per ascus, the staining properties of the ascal wall and the thickness of the ascal wall at the apex. Since in each species the apical wall narrowed markedly prior to spore release, references to the thickness of the apical walls were made at the time when the ascus was fully matured and the spores were still developing. In the species with the fewest spores per ascus, T. microsporus, the wall of the ascus was found to be thickest at the apical region. By comparison, the thickness at the apex in the multispored species, T. crustaceus and T. polysporus, was approximately the same as the rest of the ascal wall, while it was thinner than the other regions in the ascus in T. stercoreus. Consequently, as the ascus increased in spore number the relative thickness of the lateral wall became more prominent. At the same time the staining reaction of the outer layer at the apical dome was more conspicuous in the species which had fewer spores per ascus, T. microsporus and T. crustaceus, than in the species which had more spores per ascus, T. stercoreus and T. polysporus.

The ascal wall in Thelebolus was previously described to consist of three layers--a thin outer layer, a second or

middle layer, which was discontinuous and had a strong affinity for Congo red, and a third inner layer, which was selectively stained by acid fuchsin. After close inspection of one-micron sections of T. polysporus stained with toluidine blue and fresh material of T. stercoreus stained with acid fuchsin, a fourth stratum was found in the present study to line the internal region of the ascus wall.

Light microscopic observations of the apical apparatus of the Thelebolus ascus demonstrated very few microchemical and morphological similarities with the apical apparatuses of the other species found in the operculate Discomycetes. Outside of Thelebolus, occurrence of Congo red-positive apical rings has been reported only in Ascozonus (Kimbrough, 1966b, 1972; van Brummelen, 1974). In Lasiobolus monascus a mucilaginous-like ring was shown to be derived primarily from the inner layer (Kimbrough and Benny, 1977). Moreover, the ring became less prominent as the ascus approached dehiscence. On the other hand, the ring in Ascozonus was comprised mostly of a middle stratum or layer which narrowed considerably in thickness towards the apex (see Chapter II; van Brummelen, 1974). Consequently, at the light microscopic level microchemical properties of the wall layering in species of Thelebolus and Ascozonus were comparable. Nevertheless, the form of the tip of the ascus and the mode of dehiscence clearly distinguished the apical apparatuses of these taxa.

Ultrastructural examination of the Thelebolus apical apparatus demonstrated remarkable similarity among the multi-spored members. Development of the apical apparatuses in T. stercoreus, T. crustaceus and T. polysporus basically followed a three-step sequence. (1) Before the end of meiosis the external stratum of the outer layer is completely formed and the internal stratum is partially formed (Fig. 54A). Small vesicles and packets of glycogen are scattered throughout the ascus cytoplasm being concentrated toward the periphery of the cell. The ascus mother cell has grown by this time to over one-half the size of the mature ascus. (2) During subsequent mitotic divisions the internal stratum of the outer layer continues to develop in a restricted area near the tip resulting in the formation of a ring (Fig. 54C). Simultaneously, an inner layer is deposited both above and below the ring. The ascus at this time is filled with vesicles which coalesced toward the base. (3) At a slightly later time in development an internal stratum of the inner layer is formed along the entire length of the ascus (Fig. 54B). As a result the wall reaches its maximum thickness prior to spore delimitation. The ontogeny of the apical apparatuses in the multispored species of Thelebolus differed sharply with that of the apical apparatuses in operculate representatives (see Chapters I-IV). In contrast, major wall development in T. microsporus occurred considerably later during ascosporeogenesis. Deposition of the internal stratum of the outer layer was not nearly as localized in

T. microsporus. Consequently, a distinct ring was not clearly detected ultrastructurally in this species with or without the aid of silver methenamine. Pronounced thickening of the inner layer in T. microsporus was found only within the apical dome. Stratification of the wall was also less conspicuous in this species than in T. stercoreus, T. crustaceus and T. polysporus, which possessed thicker walls and greater spore number per ascus. Similar observations were made in the examination of the apical apparatus of the eugymnohymenial representative Coprotus. The apical wall of the 32-spored species Coprotus winteri (Marchal) Kimbr. was acutely more stratified than the apical wall of the 8-spored species C. lacteus (Ck. and Phill.) Kimbr. (see Chapter III).

An interesting wall feature in T. stercoreus and T. polysporus was the comparative differences of their thickness at the base. The absence of a thick wall at the base in T. polysporus sharply contrasted with the uniformly thick wall in T. stercoreus. Pores which were detected at the base of the ascus in both species during ascosporeogenesis diverged greatly in size and form. Still, the differences of the pores essentially reflected the differences of the wall thickness in that area. Within the operculate Discomycetes ascal pores have been previously reported in Ascodesmis sphaerospora Obrist, Saccobolus kernerni (Cr.) Boud. and L. monascus (Carroll, 1967; Kimbrough and Benny, 1977). In each instance a septal pore cap, which consisted of a

hemispheric layer of radiating tubules, was associated with the pore at the base of the ascus. Carroll (1967) proposed that the pore cap may act as a subcellular sieve preventing the ingress of certain cytoplasmic inclusions into the ascus while allowing the free flow of small molecules. Caps or similar structures were not found by me at the ascal pore in Thelebolus. However, two electron-dense bodies bordered the pore in the stalk cell of T. polysporus. Their role may be functionally similar to that of the septal pore cap observed in the operculate species.

The ascal walls of the species of Thelebolus in the present study differed structurally in one major aspect from those of the true operculate species currently described. Throughout the internal portion of the inner layer in the multispored species of Thelebolus stacks of microfibrils were arranged in a banded pattern. The microfibrils were uniformly upturned at the end of each stack. To a lesser extent, similar banding was observed at the apical dome in T. microsporus. In each species orientation of the bands was essentially parallel to the cytoplasm of the ascus. Fibrillar bands have been reported previously in the apical apparatuses of sarcoscyphaceous species (Samuelson, 1975) and Lasiobolus monascus (Kimbrough and Benny, 1977). However, within the inner layer of the ascal tips of suboperculate species and L. monascus, the bands consisted mostly of thick, irregular bulges which were formed for only a short distance below the operculum.



Wall structure found in Thelebolus most closely resembled that of the bitunicate ascus (Reynolds, 1971; Bellemère, 1971). The banded pattern of the secondary wall that was illustrated by Reynolds (1971) in Limacinula theae Syd. and Butl. was identical to the pattern observed in the different species of Thelebolus. Furthermore, the sequential development of the bitunicate wall in L. theae followed that of Thelebolus in several ways. During the major enlargement of the ascus, only the primary wall was formed. Deposition of the secondary or inner layer in the bitunicate and the Thelebolus ascus began before spore formation. Development of the inner layer occurred predominantly in the upper half of the ascus. The presence of two layers, each having two distinct strata in the walls of T. strecoreus and T. polysporus, was essentially the same as that described for the different bitunicate species reported by Bellemère (1971). His schematic structure of each stratum, including the banded appearance of the "inner layer of the endo-ascus," i.e., the internal stratum of the inner layer, fitted closely the structure of the wall layering within species of Thelebolus. Bellemère pointed out that the ascaltips of the different bitunicate taxa were best distinguished from one species to another by variations of the thickness of the four "layers" or strata. As in the present study, he found that most of the variations in wall thickness were restricted to the inner layer.

Although the type of dehiscence associated with species of Thelebolus has been taxonomically important, the actual mechanism involved was not known. Evidence developed by the present study has shown that asci of Thelebolus release their spores in a jack-in-the-box manner where the inner layer rapidly extends beyond the boundary of the outer layer (Fig. 54 D). Dehiscence of this kind has been found only in the bitunicate ascus. During spore discharge from a bitunicate ascus, splitting of the outer layer, or ectoascus, occurs either at the tip or circumscissely below the tip, throwing off a hood-shaped portion of the outer layer. At the same time, the inner layer expands two to three times the normal length of the ascus. Ascus dehiscence in Thelebolus appears to be a modification of this type of spore release with most of the expansion being restricted to the inner layer of the apical dome. The rigid thickening of the outer layer's internal stratum below the dome is primarily responsible for the limited expansion. Consequently, the extension of the inner layer is sharply reduced, and separation of the two layers was not visible using light microscopy.

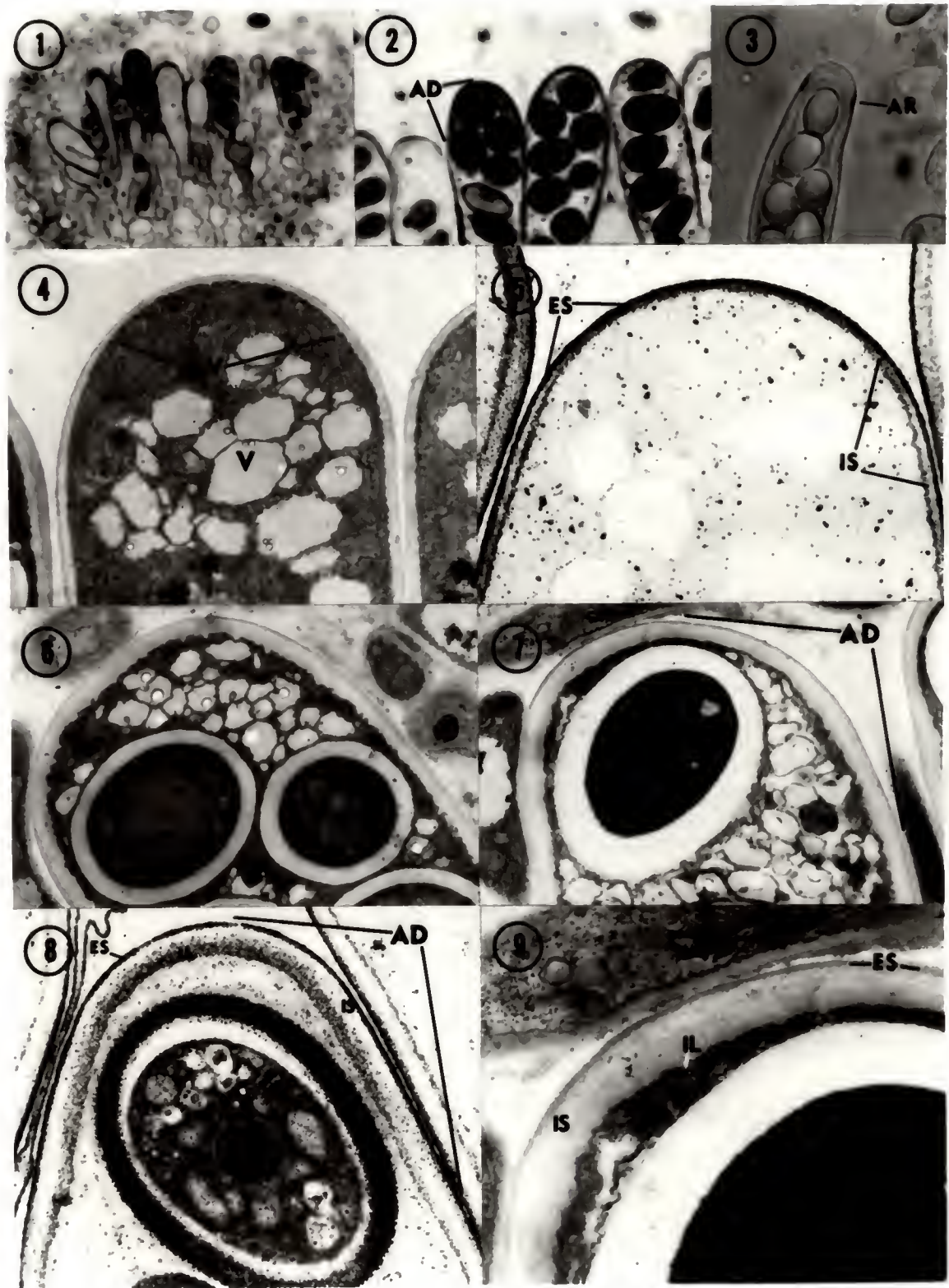
I question the taxonomic placement of Thelebolus within the Pezizales in light of the present data. Characters such as the bitunicate nature of the ascus, the jack-in-the-box type of dehiscence and the cleistohymenial development of the ascoma affiliate this genus most closely with members of the Loculoascomycetes. Further investigations concerned

with dehiscent mechanisms in the Loculoascomycetes may be useful in discovering the appropriate positioning of this genus.

## Chapter V

### Figures 1-9. Thelebolus microsporus

- Figure 1. Ascocarp shows asci at different levels of development. One-micron section stained with toluidine blue. X500.
- Figure 2. Mature asci with thickened apical wall. Apical dome (AD). One-micron section stained with toluidine blue. X1,000.
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- Figure 11. Apex of mature ascus shows apical dome (AD) and apical ring (AR). Stained with Congo red. X1,000.
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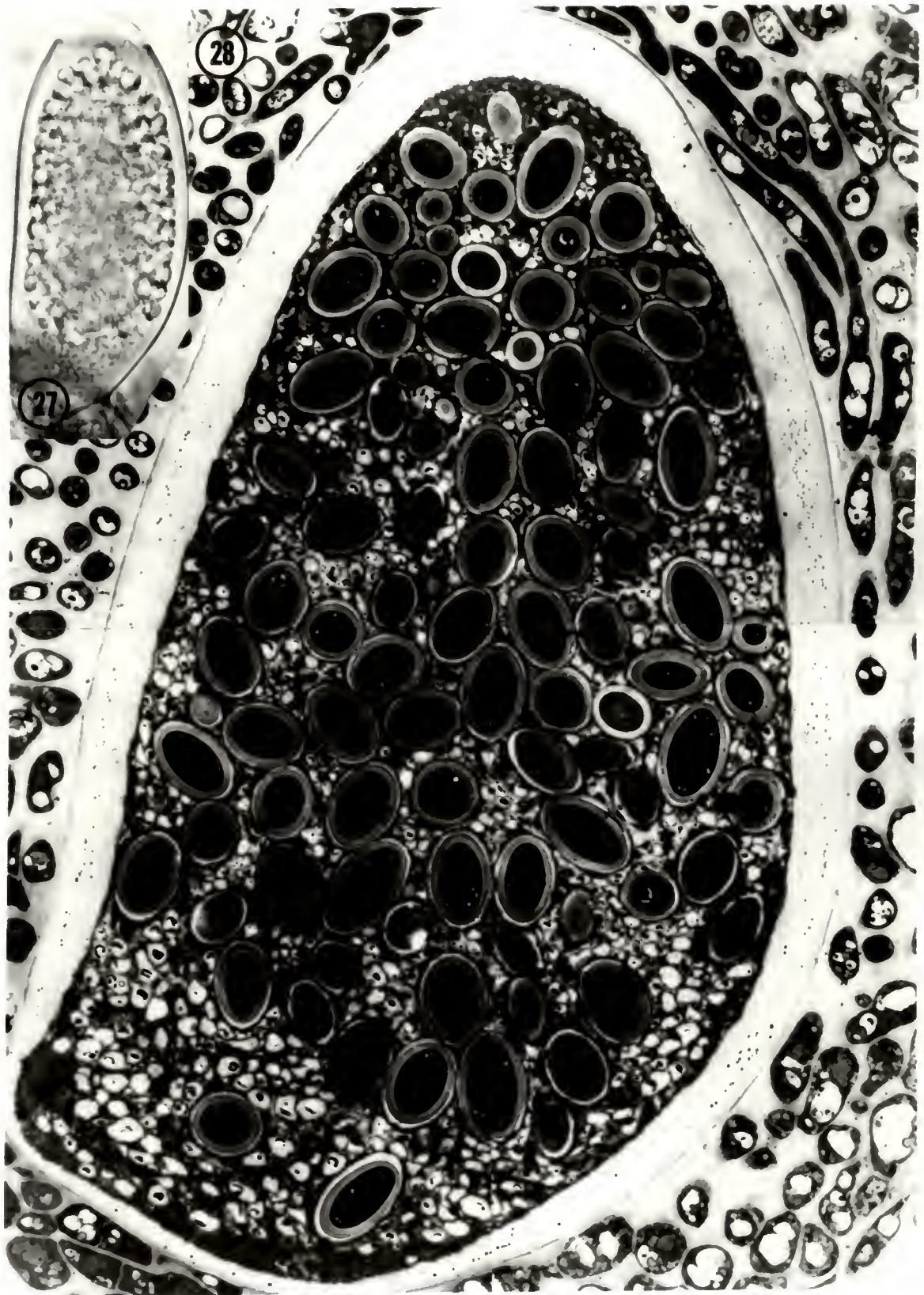
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Figures 27-28. Thelebolus polysporus

Figure 27. Mature ascus with approximately 250 ascospores. Stained with Congo red. X400.

Figure 28. Mature ascus with fully developed apical apparatus. X3,000.





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- Figure 29. Apical region of mature ascus shows apical dome (AD) and apical ring (AR). Stained with Congo red. X1,000.
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- Figure 31. Apex of mature ascus stained with silver methenamine. Apical dome (AD). Apical ring (AR). X4,700.
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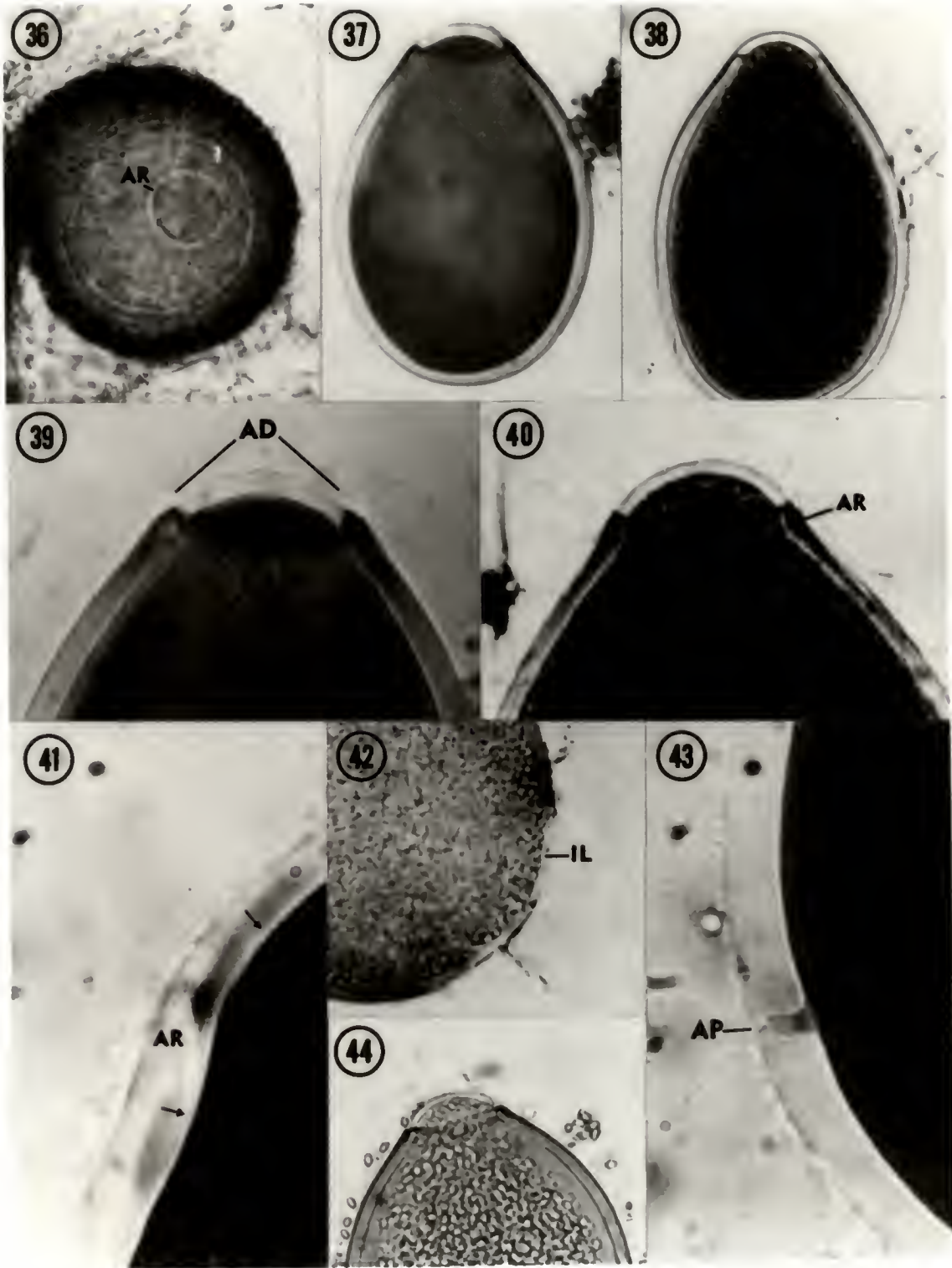




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- Figure 36. Young, multinucleated ascus showing apical ring (AR). Viewed face-on. Stained with Congo red. X320.
- Figure 37. Ascus at later developmental stage stained with Congo red. X260.
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Figure 45. Mature ascus of Thelebolus stercoreus shows thick wall throughout its entire length.  
X1,000.

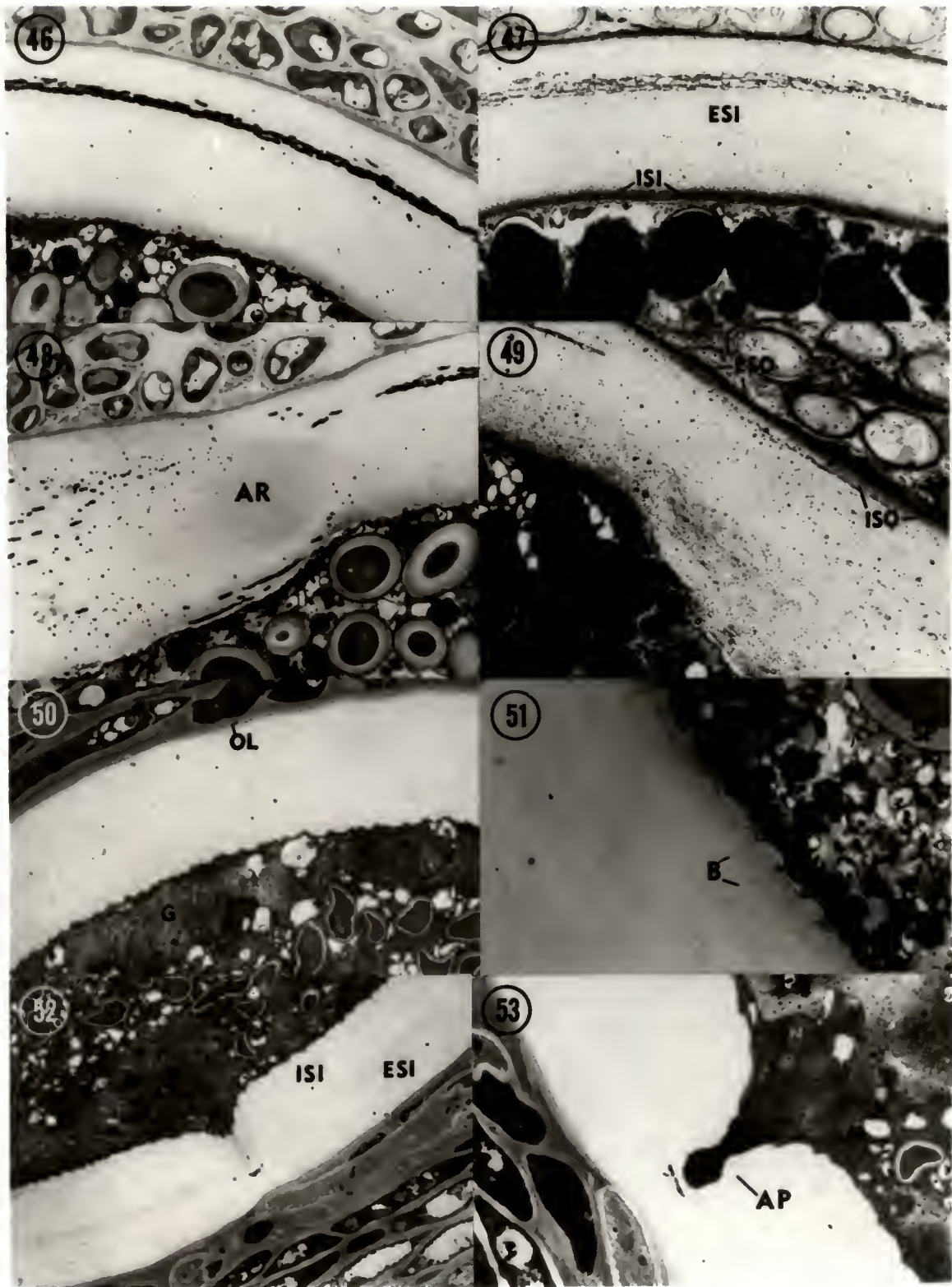


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### Figures 46-53. Thelebolus stercoreus

- Figure 46. Portion of apical dome of mature ascus shows split within inner layer. X5,300.
- Figure 47. Portion of apical dome stained with silver methenamine consists mostly of external stratum (ESI) and internal stratum (ISI) of inner layer. X5,300.
- Figure 48. Area of apical ring (AR) of mature ascus shows wall narrowing markedly above the ring. X5,300.
- Figure 49. Area of apical ring stained with silver methenamine. External stratum (ESO) and internal stratum (ISO) of outer layer. X5,300.
- Figure 50. Lateral wall of ascus during early spore wall formation. Outer layer (OL). X3,900.
- Figure 51. Internal portion of mature lateral wall shows bands (B). X6,800.
- Figure 52. Base of ascus during early spore wall formation. Internal stratum (ISI) and external stratum (ESI) of inner layer. X3,100.
- Figure 53. Large ascal pore (AP) at base of maturing ascus. X5,400.



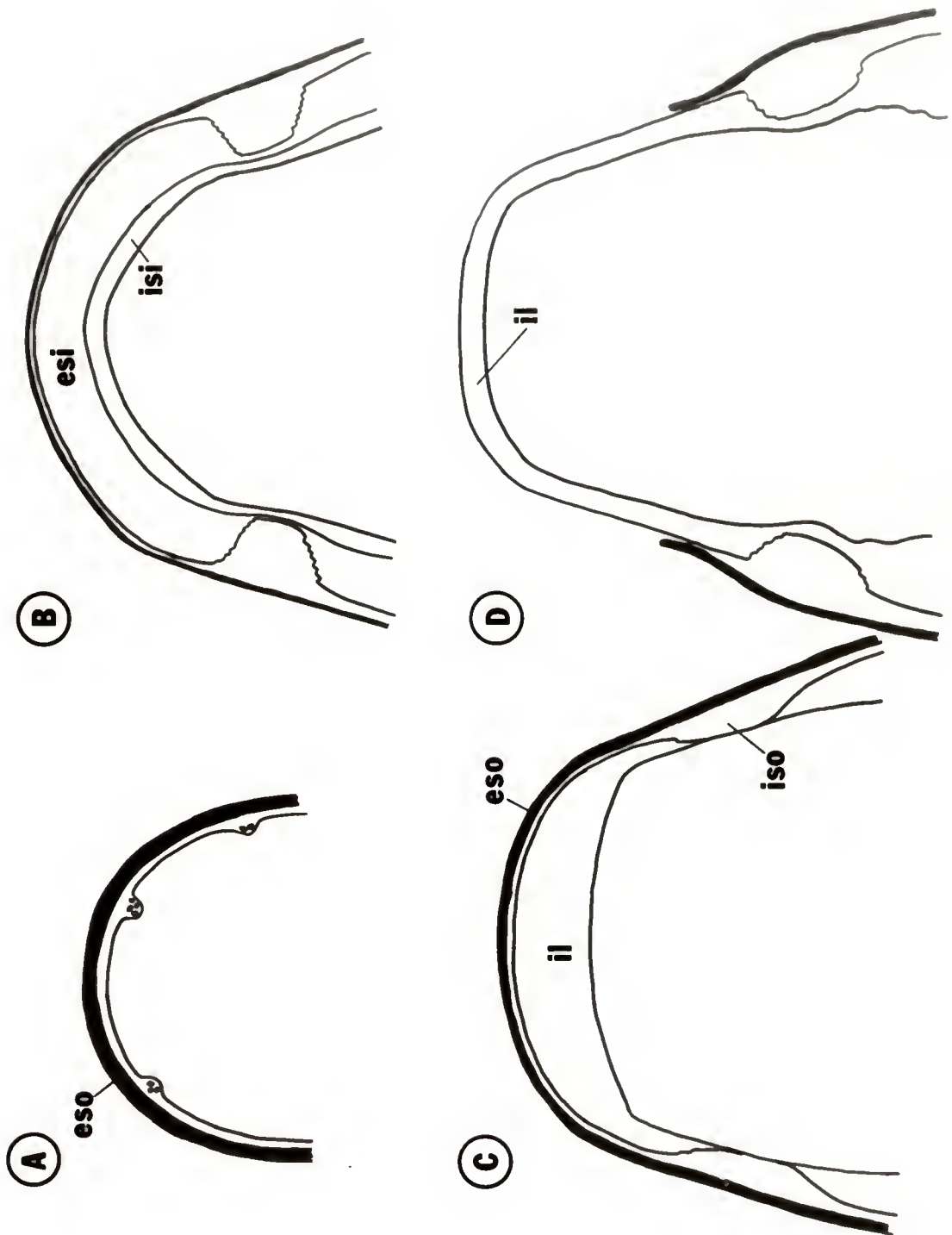


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Figure 54. Development of the apical apparatus in Thelebolus polysporus.

- A. Uninucleate ascus having thin wall. External stratum (ESO) of outer layer.
- B. Mature apical apparatus. Internal stratum (ISI) and external stratum (ESI) of inner layer.
- C. Localized deposition of inner layer (IL) at apical and lateral regions of ascus wall. Internal stratum (ISO) and external stratum (ESO) of outer layer.
- D. Extension of inner layer (IL) at apical dome during onset of spore release.





CHAPTER VI  
MORPHOLOGY, DEVELOPMENT AND CYTOCHEMISTRY OF THE APICAL  
APPARATUS OF TRICHOBOLUS ZUKALII

Introduction

A developmental study on Trichobolus zukalii Heimerl was made by Kimbrough (1966a) in order to determine the relationship of this species to other operculate Discomycetes. Heimerl (1889) had originally placed this representative in the genus Thelebolus Tode on the basis of the size and shape of the ascus, the presence of a single ascus per ascocarp, and the formation of numerous ascospores within a single ascus. Kimbrough (1966a, b) pointed out, however, that a variety of spore, ascal and apothecial characters in Thelebolus zukalii and closely related taxa differed significantly from those found in Thelebolus Tode per Fr. emend. Kimbr. Consequently, Saccardo's (1892) section Trichobolus of Thelebolus Tode was raised by Kimbrough and Cain (Kimbrough and Korf, 1967) to the generic level. The most salient features which separated species of this genus from Thelebolus were the presence of apothecial setae and the absence of an apical ring and a hyaline dome in asci of Trichobolus.

Studies of ascocarp development in Thelebolus by Wicklow and Malloch (1971) demonstrated additional support

for the removal of Trichobolus from Thelebolus. They discovered that all isolates of Thelebolus Tode per Fr. emend. Kimbr. grew best at low temperatures (5-10°C) and could be characterized as being "psychrophilic" or "thermophobic." In contrast, Trichobolus zukalii was found to be "thermophilic," having optimal growth above 25°C.

Recently, Krug (1973) placed an eight-spored species in Trichobolus and in doing so enlarged the concept of the genus to include members that formed many cylindric asci, which possessed distinct apical rings and true opercula. Furthermore, he added that the excipulum may be "dextrinoid," a character typically found in representatives of Lasiobolus Sacc. (Bezerra and Kimbrough, 1975).

Recent studies of ascal structure and its associated mechanism of spore release have made significant contributions to our understanding of the taxonomy and phylogeny within the Euascomycetes (Beckett and Crawford, 1973; Bellemère, 1975; van Brummelen, 1974, 1975; Kimbrough, 1972; Chadeffaud, 1973; Reynolds, 1971). A number of examinations on ascal structure within the Pezizineae have been made from representatives of the Thelebolaceae sensu Rifai (1968) (Kimbrough, 1966a, b, 1972; Kimbrough and Benny, 1977; Kimbrough and Korf, 1967; van Brummelen, 1975) and have aided greatly in generic delimitations within that family.

The present investigation of apical structures of operculate asci demonstrated that members of the Thelebolaceae which possessed some form of an operculum, i.e.,

Lasiobolus Sacc., Coprotus Kimbr. and Ascozonus (Renny) Hansen, resembled morphologically and developmentally the apical apparatuses of certain representatives in the Pyronemataceae sensu Korf (1973) (see Chapters II and III). Furthermore, these findings supported, in part, previous studies (Kish, 1974; Conway, 1975; Bezerra and Kimbrough, 1975) that recommended the removal of Coprotus and Lasiobolus from the Thelebolaceae.

By comparison, species of Thelebolus, which released their spores by irregular dehiscence, were shown in the present work to possess bitunicate asci. Ascal dehiscence consisted of a modified "jack-in-the-box" type (see Chapter V).

The apical apparatus of Trichobolus has been poorly understood. Spore release was reported to occur most often through an irregular tear at the ascal tip (Kimbrough, 1966a, b). Kimbrough (1966a) did note occasionally opercular dehiscence. In the enlarged concept of Trichobolus, Krug (1973) referred to ascal dehiscence in the different species of Trichobolus as being operculate. He apparently emphasized the dehiscent mechanism of the eight-spored species which he proposed was an adaptation for more controlled spore discharge.

This study examines the morphology and cytochemistry of the ascal wall of Trichobolus zukaii and its associated mechanism of dehiscence in order to understand better the taxonomic relationship of this taxon with other members of the operculate Discomycetes.

## Materials and Methods

### Collections of Material

Trichobolus zukalii was obtained from the Centraal Bureau Voor Schimmelcultures, no. 720.69. Apothecia were grown on DOA (Dung-Oatmeal Agar; Benedict and Tyler, 1962).

### Procedure for Light Microscopic Observations

Whole ascocarps of T. zukalii were squash-mounted in Congo red (Samuelson, 1975) to stain the layers of the ascal wall. Plastic embedded material was sectioned, mounted and stained in the manner described in Chapter I.

### Procedure for Electron Microscopic Observations

Five-millimeter blocks of agar, each containing 10-20 apothecia, were fixed in a buffered (0.2M sodium cacodylate pH 7.2) 2.0% glutaraldehyde and 2.0% paraformaldehyde solution for 12 hours at room temperature. The blocks were rinsed, postfixed, dehydrated, embedded and poststained as described in Chapter I.

## Results

The mature ascus of T. zukalii is ovoid to pyriform, being 300-425 x 325-510  $\mu\text{m}$  and contains 6000-7000 ascospores (Fig. 1). The ascal wall appears to be thinnest at the apex (Figs. 2, 3). When stained in Congo red, wall layering at the apex is difficult to distinguish (Fig. 2). However, when one-micron sections are stained with toluidine blue, an intensely stained inner layer is clearly demarcated from



a mildly stained outer layer at the tip and throughout the ascal wall (Figs. 3, 6). Both layers increase in thickness toward the lateral region (Fig. 3). In Congo red, the outer layer of the lateral wall is comprised of two strata with the internal stratum being more strongly stained than the external stratum (Fig. 4). The thickened inner layer remains unstained. The outer layer does not appear to be stratified when stained with toluidine blue (Figs. 3, 6).

At the base of the ascus the thickness of both layers diminishes considerably (Figs. 5, 6). Even with the aid of Congo red the two strata of the outer layer are difficult to distinguish in this region (Fig. 5).

During spore liberation the apex of the ascus occasionally undergoes a circumscissile rupture, forming a wide operculum (Fig. 7). The operculum remains fastened to one side of the ascus, appearing as an attached lid. Ascal dehiscence occurs frequently at the tip by random, irregular tearing.

Ultrastructurally, the mature ascal wall does not appear to possess a distinct apical apparatus (Figs. 8, 10, 11, 13). Sections that are stained with uranyl acetate and lead citrate do not demarcate clearly the wall layering throughout the ascus (Figs. 11, 12). Silver methenamine staining enhances markedly the visibility of layering in the wall (Figs. 8-10, 13-18). At the apical region of the wall (Fig. 8), the outer layer is composed of two strata. The external stratum is 0.55-0.60  $\mu\text{m}$  thick and appears to have

several substrata that lie adjacent to the internal stratum (Fig. 13). The internal stratum and the inner layer have approximately the same thickness, 1.10-1.20  $\mu\text{m}$ . The inner layer is not as strongly stained as the outer layer at the ascal tip (Figs. 8, 13). The apical wall is 3.00-3.25  $\mu\text{m}$  thick and increases to 4.4-5.0  $\mu\text{m}$  at the lateral region of the ascus (Figs. 10, 15, 16). Transverse sections of the ascal tip distinguish the two layers more distinctly (Fig. 17). Furthermore, the outer layer is seen to be strongly ribbed. In the upper extremities of the lateral wall the external stratum of the outer layer thickens to 0.95-1.00  $\mu\text{m}$  (Fig. 15). Delimitation of the internal stratum and the inner layer is extremely difficult in this area of the ascal wall. However, toward the lower extremities of the lateral wall, the inner layer and the two strata of the outer layer are easily distinguished (Fig. 16). The thickness of the wall at the base of the ascus diminishes to 2.60-2.95  $\mu\text{m}$  (Fig. 14). The outer layer's external stratum thickens to 1.25-1.40  $\mu\text{m}$  while the internal stratum tapers to 7.5-8.0  $\mu\text{m}$ . The inner two strata of the inner layer are stained strongly by the silver methenamine (Figs. 9, 14, 18). By comparison, substrata are not sharply detected in the external stratum of the outer layer. Scars of the attachment cells still remain in the outer layer at the base (Fig. 18).

### Discussion

Wall layering of mature asci in Trichobolus zukalii which had been stained with Congo red concurred completely with Kimbrough's (1966a) observations. The wall appeared to be comprised of three layers in the lateral and lower regions of the ascus. One-micron sections stained with toluidine blue and thin sections stained with silver methenamine indicated, however, that only two layers exist. The middle or third layer observed in Congo red was most likely the internal stratum of the outer layer which thickened conspicuously toward the base. Ultrastructural examination of the inner layer at the lower region of the ascus currently revealed the presence of three strata and confirmed earlier findings made by Kimbrough (1966a).

The mature ascal wall of T. zukalii differed primarily from that of the operculate representatives discussed in Chapters I-IV in the amount of stratification found in the outer and inner layers. The heavily stratified wall in T. zukalii contrasted sharply with the walls of other taxa including species that have more than eight spores per ascus. Furthermore, most of the stratification in T. zukalii occurred at the base of the ascus while in the other operculate species sublayering of the wall was most distinct in the area of the apical apparatus.

The apical apparatus of T. zukalii shared a number of properties with those of the operculate species studied by

me. The prominence of the outer layer throughout most of the ascal wall except at the apex in T. zukaii was a common feature in most operculate asci. T. zukaii followed the trend observed in other operculate taxa where multisporous species (as defined in Chapter V) develop thick, stratified ascal walls. The marked increase of the thickness of the ascal wall during spore formation reported by Kimbrough (1966a) strongly resembled the pattern of development found in most operculate apical apparatus (see Chapters I-IV). Ascal dehiscence occurred by the circumscissile rupture of the apex occasionally in the multisporous species and typically in the eight-spored species, T. octosporus (Kimbrough, 1966a; Krug, 1973). The infrequency of operculate dehiscence in species with large, numerous spored asci indicated that this mechanism of spore release may be functionally inadequate. The irregular tearing of the apex during the ejection of the spores was most likely a modified form of operculate dehiscence. The undulate or ribbed outer layer of the apical wall possibly aided in the uneven tearing of the tip. A similar modification was observed in Ascozonus woolhopensis (Ber. and Br. apud Renny) Hansen, which had an apical disc that was essentially inoperative during spore release (see Chapter II).

Although Trichobolus has been associated closely with Thelebolus (Kimbrough, 1966a, b; Kimbrough and Korf, 1967), having been once placed within the latter genus, the ascal wall and the apical apparatus in T. zukaii do not resemble

those in Thelebolus described in Chapter V. The absence of an apical dome subtended by a Congo red-positive ring, a bitunicate wall and a "jack-in-the-box" type of dehiscence clearly distinguished the apical apparatus of Trichobolus from that of Thelebolus and supported strongly the separation of these genera.

In terms of what is known to date, the type of apical apparatus found in T. zukaii may be regarded as unique in both function and structure. The lack of distinctive features such as an annular indentation, a subapical ring, or a differentially stained zone of dehiscence failed to demonstrate a direct alliance with the different forms of apical apparatuses in the present study. Since ascospore and apothecial characters of Trichobolus were found to be most similar to Lasiobolus, Krug (1973) proposed that T. octosporus Krug may represent a transitional form between these genera. Examination of the apical apparatus in eight-spored species of Trichobolus and Lasiobolus may reveal the degree of their relatedness. The apical apparatus of the large uniascal species L. monascus Kimbr. (Kimbrough and Benny, 1977) and T. zukaii exhibit no significant similarities. The present evidence does not support Krug's proposal.



## Chapter VI

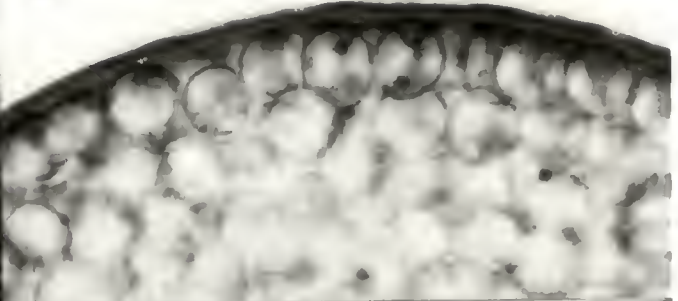
### Figures 1-7. Trichobolus zukalii

- Figure 1. Mature ascus containing 6,000-7,000 ascospores. Stained with Congo red. X280.
- Figure 2. Ascal tip shows narrowed wall without distinct layers. Stained with Congo red. X1,000.
- Figure 3. Ascal tip showing inner layer (IL) and outer layer (OL). One-micron section stained with toluidine blue. X500.
- Figure 4. Deeply stained outer layer (OL) with two strata and unstained inner layer (IL) of lateral wall. Stained with Congo red. X1,000.
- Figure 5. Base of ascal wall shows inner layer (IL) and outer layer (OL). Stained with Congo red. X1,000.
- Figure 6. Base of ascus. One-micron section stained with toluidine blue. X500.
- Figure 7. Dehisced ascus with operculum (O) partially attached. X500.

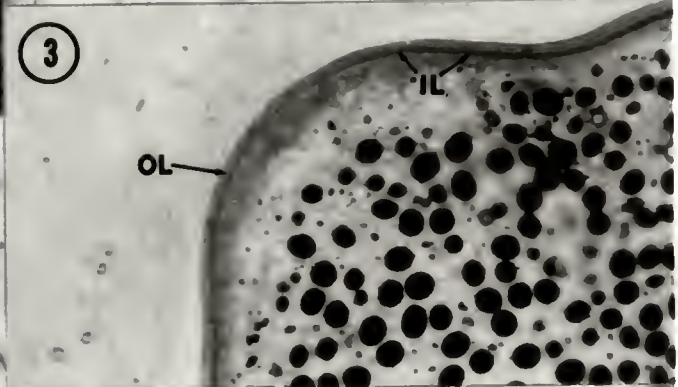
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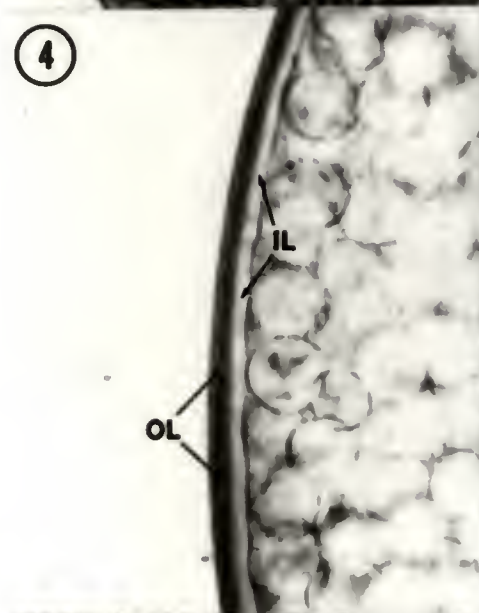
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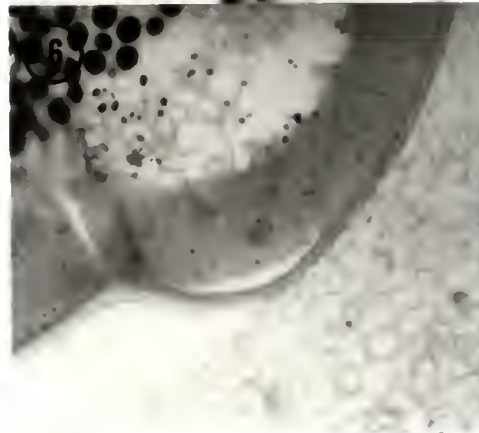
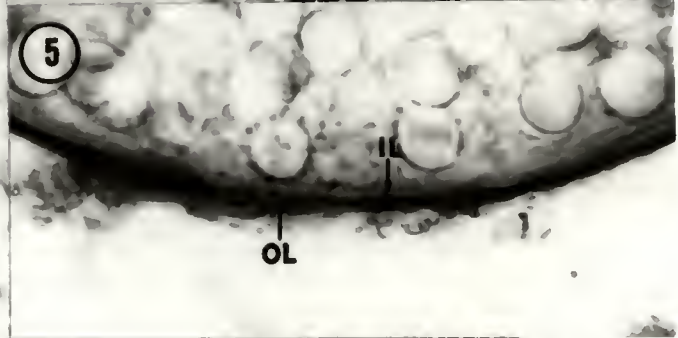
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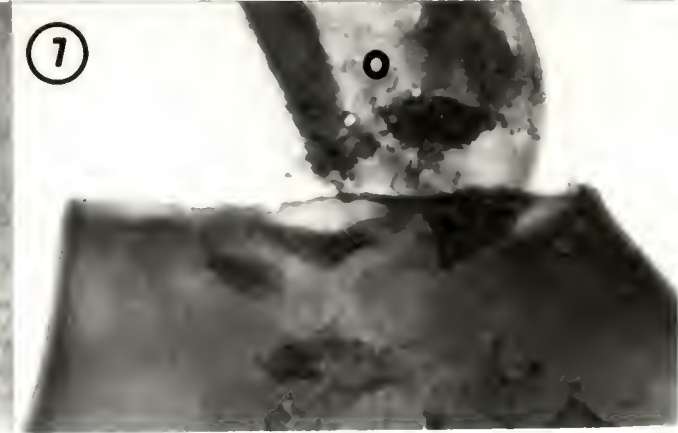
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## Chapter VI

### Figures 8-10. Trichobolus zukaii

Figure 8. Ascal apex stained with silver methenamine. Inner layer (IL). Outer layer (OL). X3,100.

Figure 9. Base of mature ascus stained with silver methenamine. Inner layer (IL). Outer layer (OL). X2,000.

Figure 10. Distal region of lateral wall. Stained with silver methenamine. Inner layer (IL). Outer layer (OL). X2,000.



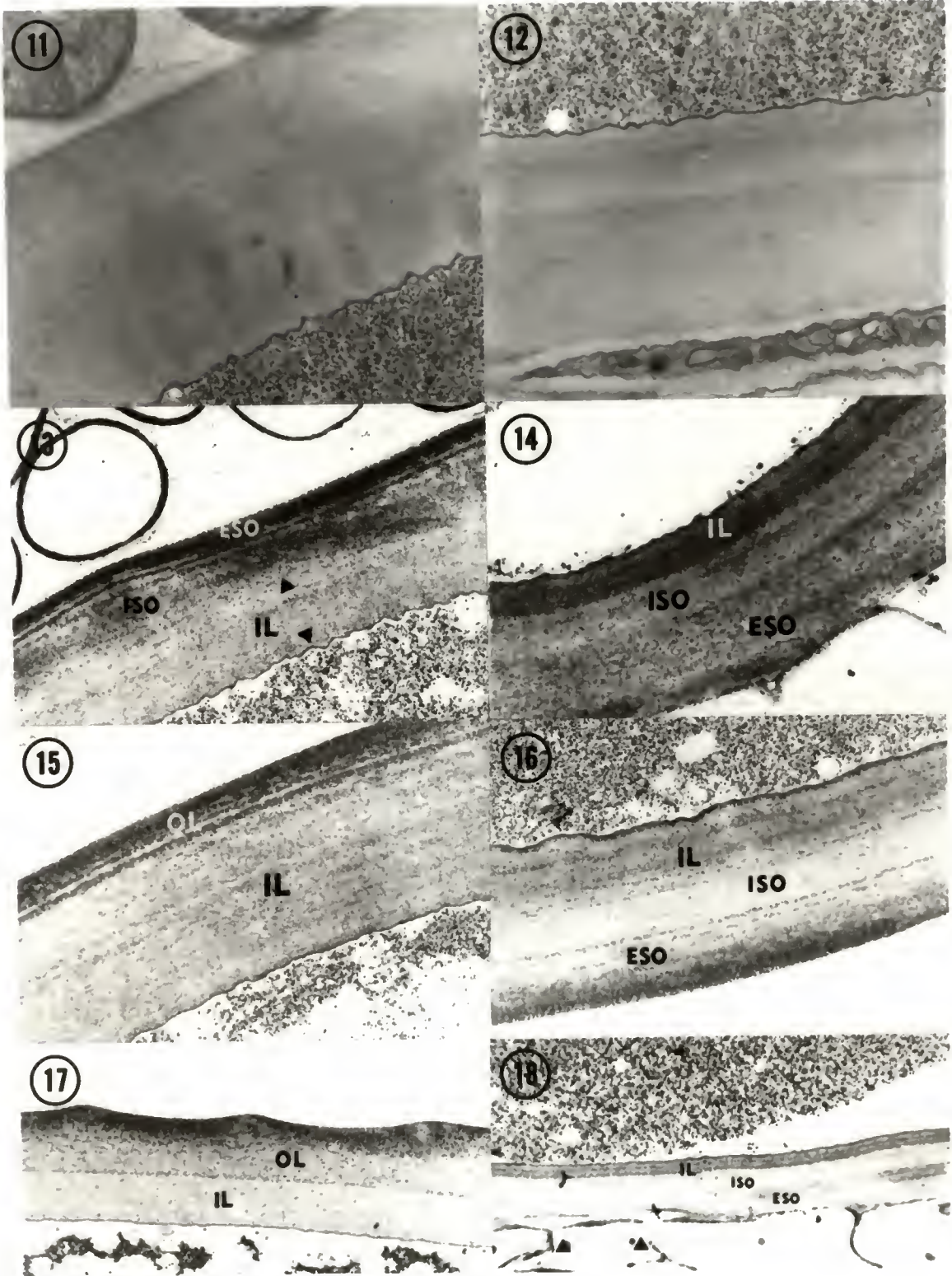


## Chapter VI

### Figures 11-18. Trichobolus zukalii

- Figure 11. Portion of apical wall. X12,000.
- Figure 12. Wall at base of ascus. X12,000.
- Figure 13. Apical wall stained with silver methenamine shows external stratum (ESO) and internal stratum (ISO) of outer layer. Inner layer (IL). X8,400.
- Figure 14. Base of ascal wall with stratified inner layer (IL). Internal stratum (ESO) and external stratum (ESO) of outer layer. Stained with silver methenamine. X,9000.
- Figure 15. Upper portion of lateral wall. Outer layer (OL). Inner layer (IL). Stained with silver methenamine. X7,600.
- Figure 16. Lower portion of lateral wall. Inner layer (IL). Internal stratum (ISO) and external stratum (ESO) of outer layer. Stained with silver methenamine. X7,600.
- Figure 17. Transverse section of ascal tip shows ribbed outer layer (OL). Inner layer (IL). Stained with silver methenamine. X6,100.
- Figure 18. Base of ascus. Arrows point to scars of attachment cell. Inner layer (IL). Internal stratum (ISO) and external stratum (ESO) of outer layer. Stained with silver methenamine. X3,900.





## SUMMARY

The importance of ascal dehiscence in the classification of Discomycetes and the Ascomycetes, in general, has long been an interesting question. By 1940, dehiscence mechanisms were considered to be reliable criteria upon which to base large group relations in the Ascomycetes. Ingold (1933, 1939) demonstrated that there was considerable variation in ascal structure in the Pyrenomycetes. Consequently, Luttrell (1951) stated "if greater emphasis were placed on ascus structure and more extensive data obtained, these variations might prove to be criteria of fundamental importance in the classification of the Ascomycetes."

The present study has demonstrated distinct variability of the operculate ascus and its apical apparatus in morphology, cytochemistry and development. Several major forms of the apical structures were observed. Members typically with iodine-positive asci, which included representatives of Ascobolus, Saccobolus, Thecotheus and Peziza, possess annular indented zones of dehiscence. Furthermore, exogenous mucilaginous coats are found in those species, which also stain blue in Melzer's Reagent. The mucilaginous coats seem responsible for the staining reaction. The apical apparatus of Iodophanus granulipolaris is the only exception. The

absence of a detectable mucilaginous coat and indented ring, along with the presence of a differentially stained opercular wall and zone of dehiscence, sharply distinguish the apical apparatus of this species from the rest of the "iodine-positive" species.

Cytochemical demarcation of the operculum and the dehiscent zone was also observed in species of Coprotus, Ascodesmis and Pyronema. The apical apparatuses of these eugymnohymenial discomycetes represent a second major form and differed from I. granulipolaris in several ways. Asci of Coprotus, Ascodesmis and Pyronema are not iodine-positive in Melzer's reagent. Their ascal walls tapered in thickness at the tip whereas the tip in Iodophanus remained as thick as the rest of the wall. Bands or wide zones of dehiscence were seen in Coprotus and Ascodesmis. In contrast, the dehiscent zone of I. granulipolaris was composed of a small swollen ring. Differential staining of the apical wall of I. granulipolaris occurred only within the inner layer. By comparison, the outer layer was the area differentially stained in the ascal tips of the eugymnohymenial species.

Additional cytochemical demarcation was found in the apical apparatus of Rhizina undulata, Helvella crispa, Morchella esculenta and Sphaerosporella brunnea. In H. crispa and M. esculenta, which possessed the third form of apical apparatus, differential staining of the ascal tips was restricted to dehiscent zones similar to those in A. sphaerospora but here were being associated with the inner

layer. A middle stratum of the opercular wall was differentially stained in the ascal tips of R. undulata and S. brunnea. Until the present study, differentially stained opercula had been found in certain representatives of the Sarcoscyphineae (Samuelson, 1975). The present findings and those of the suboperculates suggest chemical changes of the wall at specific areas, which may be specifically involved in spore ejection.

Another form of apical apparatus was observed in members of the Otidea-Aleuria complex. The lack of conspicuous dehiscent zones and the occurrence of subapical rings distinguished their ascal tips from the rest of the operculate discomycetes. Furthermore, the morphological variation of apical structures in the Otidea-Aleuria complex correlated with the marked diversity of taxa placed in this group.

The examination of the different operculate apical apparatuses in the present study supported, in general, chemotaxonomic and cytological investigations on representatives of the Pezizales made by Arpin (1968) and Berthet (1964). Berthet transferred the genera Otidea Fuckel, Sepultaria (Cooke) Lamb. and Pustularia Fuckel emend Boud. out of the Aleuriaceae sensu LeGal (1947) or Pezizaceae sensu Dennis (1968) (see Table 1, Chapter II) into the Humariaceae sensu LeGal on the basis of uninucleate paraphyses. The pronounced differences of the apical apparatuses in Peziza succosa, Otidea leporina and Aleuria aurantia strengthened Berthet's findings. Similarly, the transference



of the tribe Discineae from the Aleuriaceae sensu LeGal to the Helvellaceae by Berthet was supported by the similarities of the apical apparatuses in Helvella crispa, Gyromitra rufula and Discina ancilis and their differences with the apical apparatus in P. succosa.

Arpin (1968) found that members of the tribes Ciliarieae and Humarieae of the Humariaceae sensu LeGal possessed either both or one of two kinds of carotenes. Consequently, he combined the two tribes and placed them in a new family, the Aleuriaceae, which is shown in Table 1 of Chapter II under Aleuriaceae sensu Kimbrough. The tribe Lachneae of the Humariaceae lacked carotene and was placed in the Otideaceae sensu Eckblad (1968), which Arpin emended. Comparative analysis of different apical apparatuses of the Otidea-Aleuria complex in Chapter II supported to some extent the conclusions made by Arpin. The apical apparatuses of O. leporina, Humaria hemispherica, S. brunnea and Jafnea fusicarpa, which would be placed in the Otideaceae Eckblad emend. Arpin, generally consisted of thick to thin opercular walls and thick subopercular walls with reduced annular swellings. By comparison, the apical apparatuses of Aleuria aurantia, Anthracobia melaloma, Scutellinia scutellata, Sowerbyella imperialis and Geopyxis majalis, which would be placed in the Aleuriaceae Arpin, possessed mostly thin opercular and subopercular walls, often with conspicuous subopercular rings. The formation of two groups of apical apparatuses within the Otidea-Aleuria complex, however, was



found to be impractical after closer inspection. Differences in the structure of the apical wall were more pronounced between J. fusicarpa and S. brunnea than between A. melaloma and S. brunnea. The apical apparatus of the Otidea-Aleuria complex collectively represented a wide spectrum of one major form of operculate dehiscence.

Members of the Pezizales have been characterized chiefly by the operculate dehiscence of their asci. Species of cup fungi that release their spores by the dissolution of an apical plug or in a jack-in-the-box manner have been placed in the inoperculate Discomycetes and the Loculoascomycetes, respectively. However, within the Pezizales, a group of representatives that eject their spores through a variety of dehiscent mechanisms including operculate apparatuses has remained. They are placed in a single family, the Thelebolaceae sensu Rifai (1968), on the basis of similar ascal, ascospore, apothecial, and habitat characters. Recent investigators (Kish, 1974; Conway, 1975; Bezerra and Kimbrough, 1975) proposed that Lasiobolus and Coprotus, the only two functionally operculate genera of the Thelebolaceae, should be transferred to the Pyronemaceae sensu Eckblad (1968). The present study supported their proposals and also pointed out that the taxa, Ascozonus and Trichobolus, which form nonoperative opercula, show closer affinities with species of the Otidea-Aleuria complex than with the nonoperculate representatives of the Thelebolaceae.

Two genera, Mycoarctium Jain and Cain (Jain and Cain, 1973) and Lasiothelebolus Kimbr. and Luck-Allen (Kimbrough and Luck-Allen, 1974), have been added to the Thelebolaceae since 1970. Both genera have setose apothecia with non-operculate, eight-spored asci. Of the five nonoperculate genera currently placed in the Thelebolaceae, only Thelebolus and Lasiothelebolus appeared to be closely related to each other. Although a critical examination of Lasiothelebolus has not been made, light microscopic observations of the apical apparatus demonstrated microchemical and morphological similarities with that of Thelebolus (Kimbrough and Luck-Allen, 1974). Species of Thelebolus were shown in Chapter V to have bitunicate asci and jack-in-the-box type of dehiscence. On the basis of these and other characters including ascal and apothecial development, Thelebolus and most likely Lasiothelebolus do not belong in the operculate Discomycetes. Similarly, Caccobius, with its inoperculate-like apical apparatus, and Mycoarctium, with its evanescent asci, most likely should not be placed in the Pezizales. The mechanism of ascal dehiscence in Coprobolus is not understood. Cain and Kimbrough (1969) described the formation of a bilabiate split at spore liberation. Like Ascozonus, the apical apparatus of Coprobolus may have a modified, non-operative operculum. Further examination of this taxon is needed in order to determine whether it should be placed in the Pezizales.

After close inspection of a large number of coprophilous species distributed among the Discomycetes, Pyrenomycetes and Loculoascomycetes, Kimbrough (1972) noted that a variety of adaptations occurred. He pointed out that there was a tendency toward an increased spore number per ascus, accompanied by a reduction of asci per ascocarp. This trait was conspicuous in members of the Thelebolaceae which possessed multispored species in most genera. In the developmental study of Coprotus lacteus, Kish (1974) felt that the multispored tendency could be ignored phylogenetically because this trait was probably an ecological adaptation. Evidence in the present study supports his conclusion. I believe that only representatives which have functional or nonfunctional operculate apical apparatuses should be retained in the Pezizales. The term nonfunctional is referred to here as that which has the capacity to form an operculum but does not function normally in an operculate manner during spore release.

The taxonomic placement of Thelebolus and Lasiothelebolus is in serious doubt. As previously mentioned, current findings strongly favor the transfer of these genera to the Loculoascomycetes. Unfortunately, among the Loculoascomycetes unicellular, hyaline-spored representatives (the Botryosphaeriaceae) are neither coprophilous nor multispored, and representatives that are coprophilous (the Sporormiaceae) are neither hyaline-spored nor multispored, although most members do have septate ascospores. On the basis of ascal

and ascocarp development, Thelebolus would appear to be most closely related to the Pleosporales. Further investigations concerned with dehiscence mechanisms in the Pleosporales may be useful in discovering the appropriate positioning of these genera.

Most Ascomycetes release ascospores in a violent and consistent manner. Forceful discharge occurs principally through either a pore, an operculum or a jack-in-the-box manner. In addition, modifications of each type of dehiscence mechanism have been observed. The inoperculate, operculate and bitunicate apical apparatuses share three important features. First, they are bilayered. Frequently, sublayers or strata are associated with one or both layers, and distinction of the two layers may be extremely difficult unless the wall is studied developmentally. Second, the mature ascus becomes conspicuously stretched prior to spore release. The wall of the apical apparatus narrows considerably in thickness during this stage. At the same time the spores become tightly lodged against the inner face of the apical apparatus. Third, the spores are squeezed by the ascus wall surrounding the ascostome during dehiscence. This supplies an added impetus to each spore as it leaves the ascus. As a result, the operculate, inoperculate, and bitunicate apical apparatuses functionally behave in a similar manner.

By comparison, the three major types of apical apparatuses differ basically in structure. Furthermore, these

differences directly reflect the manner of their dehiscence. Ultrastructural investigations of different representatives of the Pyrenomycetes and inoperculate Discomycetes revealed that the pored apical apparatus consists primarily of an apical cushion, an apical ring (=pore cylinder or annulus) and a manubrium (=pore plug or axial body). Studies of Ciboria acerina Whetz. and Buchw. (Corlett and Elliot, 1974), seven pyrenomycetes (Griffiths, 1973), Xylaria longipes Nitschke (Beckett and Crawford, 1973), and Sordaria fimicola (Reeves, 1971) demonstrated that the ascal apex is considerably less complex than previously interpreted from light microscopic observations (Chadefaud, 1942). However, in Bulgaria inquinans Fr. Bellemère (1969) described greater morphological complexity. Having based his terminology on that of Chadefaud (1960), he referred to a superior ring, an apical cap, and an apical dome in addition to the apical cushion, apical ring, and manubrium. Nevertheless, the pored apical apparatuses of different taxa have generally shown various elaborations of the basic construction.

The bitunicate ascus is characteristically composed of a thick apical and lateral wall. The apical apparatus of the bitunicate ascus is not as clearly defined as that of the inoperculate ascus. An ocular chamber or pore is often formed immediately below the apex and is usually encased by longitudinal striations that are referred to as the nasse. Bellemère (1971) found that the wall was organized into two pairs of sublayers or strata with one pair comprising the



inner layer and the other pair comprising the outer layer. The inner layer contains banded microfibrils that are horizontally oriented in the plane of the ascal wall. Reynolds (1971) demonstrated that in the apical wall, margins of the bands often appear as linear striae which are visualized in light microscopy as a "nasse apicale" (Chadefaud, 1942). In general, the organization of specific components in inoperculate ascal tips and their manner of dehiscence, i.e., the dissolution of a pore plug inside of an annulus, followed by eversion of the pore, clearly distinguish the pored apical apparatus from the bitunicate type.

In studying the various apical structures of asci in the Discolichens, Chadefaud (1973; et al. 1969) called attention to the broad spectrum of forms found in that group. Certain members formed bitunicate asci which did not release their spores in the jack-in-the-box manner. Some members possessed rings within their apical apparatuses as well. On the basis of these and other findings among the different taxa of the Discolichens, Chadefaud proposed that the pored or annelaceous ascus and the bitunicate or nassasceous ascus evolved from a common ancestor which shared both characters and that the operculate ascus was derived from the pored ascus. He and LeGal (1946b) believed that members of the Sarcoscyphineae represented an intermediate line of evolution between inoperculate and operculate species. Samuelson (1975) and van Brummelen (1975), however, clearly demonstrated

that sarcoscyphaceous taxa were truly operculate and did not bridge the Pezizales and the Helotiales.

The operculate ascal tip is the most distinctive of the three major types of apical apparatuses, consisting of an operculum, a zone or line of dehiscence, and a suboperculum. In addition, operculate tips are generally thin-walled and conspicuously bilayered. Like the bitunicate ascus, the inner layer of the operculate ascus is most pronounced at the apex. The development of the inner layer, however, occurs much later in the operculate ascus than in the bitunicate ascus. In contrast, layering of the pored apical apparatus has been difficult to determine. Codron (1973) and Bellemère (1975) proposed that the annulus, manubrium and apical dome were derived from the inner layer. Greenhalgh and Evans (1967) basically agreed in their findings but hesitated to state specifically whether a particular component was formed by either layer. Corlett and Elliott (1974) pointed out that although the outer layer remained thin at the tip of the pored ascus, the inner layer was also being thinly deposited in the lower region of the apical dome and manubrium. They concluded that most of the apical dome and manubrium was developed from a middle layer which they called ground material and that the annulus appeared to have originated from the outer layer. Further cytochemical and developmental examination of inoperculate asci is needed to resolve this question. In any event, the pronounced differences in the construction and development

of the three types of apical apparatuses do not support the theoretical schemes of Chadeffaud (1942, 1964, 1973) which are concerned with the origin and evolutionary trend of apical apparatuses found in the Ascomycetes (see General Introduction). At present there is no evidence to suggest that the operculate ascus was derived from either the bitunicate or the inoperculate ascus.

The operculate asci that I examined had a single ascal wall, which structurally consisted of two layers. Chadeffaud (1942) similarly described asci, which have pored and jack-in-the-box types of dehiscence, as having single bilayered walls. Luttrell (1951), however, divided asci into two major types, bitunicate and unitunicate, on the basis of wall structure. The bitunicate type incorporated those asci that are surrounded by two distinct walls, whereas the unitunicate type incorporated asci that have a single wall. In Lecanidion atratum (Hedw.) Endl., Butler (1939), the first worker to report the jack-in-the-box type of dehiscence in the Discomycetes, used the terms "ectoascus" and "endoascus" to designate, respectively, the outer wall and the extended inner membrane, i.e., the inner wall. Luttrell used the same terms for his description of the bitunicate ascus. In the course of evaluating recent light and electron microscopic investigations of ascal walls and apical apparatuses, Chadeffaud (1960, 1973) maintained that the ascal wall is composed of two tunica, which correspond to the term "layers" in his earlier studies. He referred to the external tunica

or outer layer as the exoascus and the internal tunica as the endoascus. In addition, he called the film (=mucilaginous coat), which occasionally covers the ascus, the ectoascus. Greenhalgh and Evans (1967) stated that to apply the terms exoascus and endoascus, as used by Chadeffaud, to the inoperculate ascal wall of Hypoxylon fragiforme (Pers. ex Fr.) Kickx. would be misleading. They believed that the ascal wall of H. fragiforme is structurally unitunicate. Beckett and Crawford (1973) further reiterated this point stating "future references to unitunicate or bitunicate asci should be made only within the limits laid down by Luttrell (1951) and that these terms are defined with regard to both structure and function." Nevertheless, ultrastructural evidence of operculate and inoperculate asci (the unitunicates) and asci with jack-in-the-box type of dehiscence (the bitunicates) in the present and previous studies (Bellemère, 1971; van Brummelen, 1974, 1975; Corlett and Elliott, 1974) demonstrated that asci generally possess a single bilayered wall. Moreover, among the operculate discomycetes, the two layers appear to be very distinct. In accordance to the interpretation of Greenhalgh and Evans, the operculate ascal wall would be structurally bitunicate. Consequently, in view of the findings concerning the fine structure of ascal walls, caution should be observed when defining and interpreting terms connected with wall structure.

My observations of operculate asci and, in the case of Thelebolus, bitunicate asci, conceptually agree with the

terminology of Chadeaud (1942, 1960, 1973). All ascal walls can be conceived as being at least structurally be-layered, and if one conceives that the term "tunica" refers to layer, all asci then are bitunicate. Only ascal walls which have separable layers, however, can be conceived as being functionally bitunicate. Furthermore, functionally bitunicate walls can be structurally characterized due to the presence of bands within the inner layer. In taxa that do not release their spores in a jack-in-the-box manner but are structurally similar to those that do, the term "nassasceous" can be used. Ascal walls which do not have separable layers nor have "nassasceous" tips can be referred to as being functionally unitunicate. As a result, the terms "bitunicate" and "unitunicate" are perfectly acceptable in a reconceptualized sense.

The present study shows that ascal structure and its associated apical apparatus have marked variability in the operculate Discomycetes. Comparative analysis of the different dehiscence structures can be useful in helping determine taxonomic affinities at special, generic and familial levels. Characters such as the apical apparatus will continue to be significant in the phylogeny and taxonomy of this group of organisms.

As the result of the present examination of the operculate apical apparatus, the following conclusions are made:

1. With the exception of Iodophanus granulipolaris, iodine-positive asci appear to be useful as a taxonomic character.



2. The reaction site of the stain, Melzer's reagent, in asci which have annular indentations is an exogenous mucilaginous coat. In I. granulipolaris the reaction site appears to be the ascal wall.
3. Among the ten representatives of the Otidea-Aleuria complex presently studied, seven form subopercular rings. For the most part, those species which have thick, 700-1000 nm, lateral walls possess less conspicuous subopercular rings than those species which have thin, 400-400 nm, lateral walls.
4. Apical apparatuses of members in the Otidea-Aleuria complex, in general, lack distinct zones of dehiscence.
5. Walls of multispored asci (more than eight spores per ascus) are typically thicker and more stratified than those of their eight-spored counterparts.
6. Morphological and cytochemical similarities of the apical apparatuses in Ascodesmis, Pyronema and Coprotus help demonstrate greater relationship between these eugymnohymenial taxa and support the belief that these taxa are most closely related to members of the Otidea-Aleuria complex.
7. Comparative analysis of the apical apparatuses in species of Helvella and Morchella suggest a greater taxonomic relationship between these members than with any other operculate group.

8. Except in Ascozonus woolhopensis major development of the operculate ascus occurs late in spore development.
9. Although ascal dehiscence is nonoperculate in Ascozonus and Trichobolus, both taxa do form true opercula, which are functionally inoperative.
10. No two genera share identical apical apparatuses.
11. In Thelebolus the ascal wall is functionally bitunicate. The ascus dehisces in a modified jack-in-the-box manner.
12. The family Thelebolaceae, which needs revision, does not belong in the Pezizales.
13. All asci with forceful spore discharge are at least bilayered. The term "unitunicate" should refer to those with an inseparable wall.

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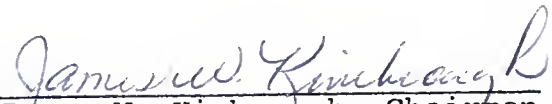
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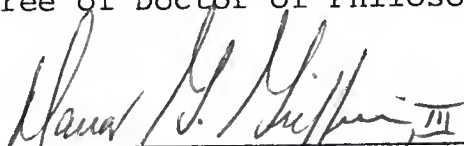
## BIOGRAPHICAL SKETCH

Don Arthur Samuelson was born August 30, 1948, in Boston, Massachusetts. He attended grammar school in Brunswick, Maine, until 1958, whereupon he moved with his parents and older brother to Newton, Massachusetts. In June, 1967, he was graduated from the Rivers Country Day School in Weston, Massachusetts. He received his Bachelor of Arts with a major in biology from Boston University at Boston, Massachusetts, in May, 1971. From 1971 to 1972 he worked as a law clerk in a corporate law firm at Boston, Massachusetts. In September, 1972, he entered the Botany Department of the University of Florida at the graduate level and earned the degree of Master of Science in March, 1975. In April, 1975, he continued to pursue his graduate studies toward the degree of Doctor of Philosophy at the University of Florida. He was married to the former Leslie Joyce Gilbert on February 14, 1977. He is a member of the Mycological Society of America, the American Phytopathological Society, the Botanical Society of America, and the Association of Southeastern Biologists.

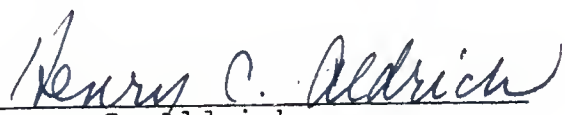
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James W. Kimbrough, Chairman  
Professor of Botany

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Dana G. Griffin, III  
Associate Professor of Botany

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Henry C. Aldrich  
Professor of Microbiology

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This dissertation was submitted to the Graduate Faculty of the Department of Botany in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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